Characterisation of Fibrinogen and Fibrin Proteolysis by the Neutrophil Membrane

by

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A thesis submitted for the degree *Doctor of Philosophy (Medicine)*

University of Cape Town

August 1999

Dedication

To my family

Acknowledgements

I would like to express my sincere appreciation to all who have contributed to the production of this thesis. In particular I would like to thank the following people.

Professor Enid Shephard, who has given so generously of her time and expertise in supervising this thesis. Her dedication to her work and to her students has been an inspiration to me and to many others who have come before me.

My father, who allowed me to work in his laboratory, and who has been such a wonderful role model both as a doctor and a scientist. His kindness, wisdom and eternal optimism guided me safely over the many obstacles of laboratory life.

Vivienne Woodburne, my first my teacher, friend and co-worker in the lab, whose commitment and contribution to this project over the years has been enormous. Her laboratory and technical expertise and attention to detail led many improvements in the design of experimental procedures used in this study. This thesis also belongs to her.

The members of UCT Electron microscope unit (EMU) who have helped me in so many ways. I am, in particular, indebted to Mohamed Jaffer for the enormous contribution that he made to this project as an electron microscopist *par excellence*, problem-solver, advisor and friend. I cannot thank him enough for all the time and energy he has spent helping me. I am also very grateful to Trevor Sewell, the head of the EMU, for taking such an active interest in my project, for treating me like a member of his unit (where I felt I had a second home), and for allowing me full access to his unit including the use of his office! I am also grateful to William Williams, Miranda Waldron and Dane Gerneke for the help that they have given me.

Joel Dave, my good friend, who has travelled the same road sharing the ups and downs of laboratory life along the way. He spent many

months helping me with the molecular biology side of my project, which he did with great patience and enthusiasm despite a busy schedule. His friendship and support has meant a great deal to me over the years.

Peter Meissner for the many ways in which he helped me with my thesis and for constantly reminding me of the lighter side of the laboratory. Nothing has ever been too much trouble for Pete and I am grateful for his generosity, friendship and the early morning coffee visits whilst I was writing up!

Mr Wells for running the laboratory so smoothly and for patiently fixing everything that I broke during my first year in the laboratory. His calm manner and his ability and to solve any problem made him the backbone our laboratory.

Brent Jennings for patiently helping me when I ran into computer problems and for his friendship over the years.

Lesley Frith and Val Hancock for keeping me supplied with purified human fibrinogen.

Anne Corrigall, Ed Sturrock, Lutz Thilo, Mario Ehlers and Pauline Hall who, despite busy schedules, gave generously of their time and expertise, in advising me on various aspects of my project.

Fikile Daki who, in addition to keeping me supplied with clean glassware and a spotless working environment, took the time to become my friend and to teach me Xhosa. Nangamso Madiba!

The many people in the laboratory who have contributed to this thesis through useful discussions, technical assistance or the donation of blood. In particular I am grateful to Susan Adams, Helen Botes, Vanessa Burch, Sandra Davids, Lester Davids, Brandon Davidson, Heather Epstein, Trevor Finch, Richard Hift, Sharon Kelly, Anwar Mall, Leslie Martin, Lavinia Petersen, Faiza Pearce, Mbulelo Maneli, Mogamat Samuels, Kwanele Siziba and Wendy Spearman.

Lastly, on a personal level, I would like to express my deep gratitude to my family. My parents have "lived" this thesis with me offering constant support and encouragement along the way, and never losing faith in me. Whilst writing up this thesis I was inspired by the beautiful Xhosa textbooks that my mother has written, from which I got many ideas on layout and design. I am also grateful to my brothers David and Derek who have been pillars of support over the years, as well as to my grandmother, my mother-in-law Pepsi, and my brother- and sister-in-law David and Debbi for all the encouragement they have given me. Thanks to Pepsi for generously supplying the paper on which this thesis is printed. Finally, I would like to thank Tanya, my wife, for the many sacrifices that she has made to enable me to undertake this project. This would not have been possible without her love, patience, support and understanding, for which I am deeply grateful. This thesis is dedicated to my family.

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Abstract

Recent studies have identified a novel 600 kDa neutrophil membrane associated protease which degrades fibrinogen, fibrin and C-reactive protein (CRP) during incubation of these ligands with phorbol 12-myristate 13-acetate (PMA, 5-10 ng/ml) stimulated neutrophils. This proteolysis is predominantly an extracellular event which occurs through a ligand dependent release of this protease from the neutrophil. Degradation products arising from this proteolysis not only become neutrophil associated but influence a number of important processes occurring in inflammation and coagulation. The aim of the present study was to purify and further characterize this protease and investigate the location of the neutrophil associated fibrinogen and fibrin degradation products.

Whilst enzyme purification procedures were unsuccessful, several observations made during these attempts suggested that the neutrophil membrane associated proteolytic activity displayed similar characteristics to proteases of the azurophil granule. The proteolytic activity of the membrane was concluded from inhibitor profiles, zymography, and the apparent molecular mass values and hydrophobicity of the fibrinogen degradation products that it generated, to be the composite action of the azurophil granule proteases, human neutrophil elastase, cathepsin G and possibly proteinase 3. Electron microscopy analysis of PMA stimulated neutrophils incorporated within fibrin clots revealed morphological changes suggestive of neutrophil degranulation, and the proteolytic activity released by these cells was shown to be identical to that of azurophil granule proteases with respect to the apparent molecular mass values of the fibrin products that it generated. Immunoelectron microscopy revealed minimal internalization of fibrin like material during this process suggesting that neutrophil mediated fibrinolysis under these conditions is predominantly an extracellular event.

Immunoelectron microscopy was used to localise fibrinogen degradation products previously reported to be associated with the neutrophil following incubation with fibrinogen. This revealed

neutrophil associated fibrinogen products to be intracellular. Internalisation appears to be the result of pinocytosis which is stimulated in the presence of PMA. Although internalisation may be enhanced by an initial interaction of fibrinogen with the neutrophil membrane, a large proportion of uptake occurs via the fluid phase. Both intact and degraded forms of fibrinogen can associate with the neutrophil. Internalised material is rapidly degraded intracellularly into low molecular weight products which are partially released into the surrounding medium. This intracellular degradation, however, contributes minimally to the overall degradation of fibrinogen by neutrophils; the major pathway is extracellular.

The demonstration in this study, that the previously identified fibrinogen- fibrin- and CRP-degrading activity of the neutrophil membrane is due to azurophil granule proteases co-incides with numerous recent reports suggesting that membrane bound forms of these proteases, due to their ability to evade naturally occurring protease inhibitors, are the biologically relevant forms of these proteases. The membrane expression of azurophil granule proteases has recently been shown to be under the control of a variety of inflammatory mediators. Thus, neutrophil mediated degradation of fibrinogen, fibrin and CRP *in vivo* may be tightly controlled by the regulated expression of azurophil granule proteases on the neutrophil membrane.

Chapter One

Introduction

Mammalian survival depends upon an innate immune system that can respond rapidly to infection without prior exposure to invading pathogens. Polymorphonuclear leukocytes (neutrophils) are central to this innate immune system and represent the first line of defence against bacterial infection.[1-3] Beyond their role as phagocytic cells, neutrophils can influence a variety of other biological processes. Their intimate association with fibrin thrombi has long been recognised,[4-6] and it has become increasingly apparent that the coagulation and inflammation pathways are intimately linked.[7,8] Leukocytes can regulate the coagulation pathway[9-15] whilst coagulation proteins can modify the inflammatory response.[16-21]

During inflammation, neutrophils accumulate in affected tissues where they engulf invading pathogens and remove necrotic debris. This activates a number of potent mechanisms capable of destroying internalised microbes which are subsequently digested by proteolytic enzymes of the neutrophil granules.[1-3] The local release of inflammatory mediators may stimulate monocytes as well as endothelial cells to express tissue factor on their cell surfaces.[22] Tissue factor triggers the coagulation cascade which culminates in the conversion of prothrombin to thrombin.[23] Thrombin cleaves fibrinogen, the essential ingredient of the fibrin clot, which then polymerises to form the insoluble gel-like matrix, fibrin.[24] Fibrin deposition is thus a frequent accompaniment of the acute inflammatory response and is thought to be important in the process of tissue repair.[25] Neutrophils have also been found in intimate association with fibrin within the vascular compartment.[4-6] Leukocytes frequently accumulate in complex thrombi, formed by fibrin and platelets, showing preferential accumulation relative to their concentration in the bloodstream.[4] The ability of leukocytes to degrade fibrin clots has been noted since the turn of the century,[26,27] and it has been suggested that neutrophils may provide an alternative

fibrinolytic pathway to that mediated by plasmin.[9] The ability of neutrophils to degrade fibrinogen and fibrin has been attributed to two proteases residing in the azurophil granules of neutrophils, namely human neutrophil elastase (HNE) and cathepsin G.[10,11,28,29] Cleavage of fibrinogen by these proteases renders it non-clottable, and generates degradation products with potent anticoagulant properties.[9,10] Such proteolysis may allow the neutrophil to regulate fibrin deposition at sites of inflammation.

Whilst neutrophils are capable of influencing the coagulation system, so too may coagulation proteins modify the neutrophil inflammatory response. Neutrophils adherent to immobilised fibrinogen exhibit a much greater response to inflammatory stimuli than non-adherent neutrophils.[16,17,30] This interaction is regulated by a family of adhesion receptors, known as integrins, of which the β_2- and β_3-integrin subfamilies can recognise specific peptide sequences on the fibrinogen molecule.[31-35] Ligation of these receptors by immobilised fibrinogen can signal for important neutrophil functions such as phagocytosis, respiratory burst and degranulation.[16,17,30,36] In addition fibrin(ogen) products released during coagulation and fibrinolysis have been shown to influence neutrophil function. Plasmin- and HNE-derived *fibrin* degradation products, as well as the thrombin cleaved fibrinopeptide B, are chemotactic for neutrophils.[20,21,37] On the other hand, plasmin derived fragments of *fibrinogen* have been reported to inhibit neutrophil respiratory burst, chemotaxis and adhesion.[18,19]

The present study was stimulated by the identification of a neutrophil *membrane* protease capable of degrading fibrinogen and solubilising fibrin clots.[13,14] This protease was shown by size exclusion chromatography to be 600 kDa in size and to be composed of at least 4 subunits when subjected to SDS-PAGE.[38] While stimulation of neutrophils with a low dose of PMA led to a partial release of this protease into the extracellular medium, incubation of intact neutrophils with substrates for this enzyme induced a time dependent egress of this proteolytic activity from the membrane.[13,14,38] The proteolysis of fibrinogen and fibrin by this membrane associated protease was shown to have several important effects on both the coagulation and inflammatory responses. Proteolysis of fibrinogen

rendered it non-clottable through cleavage between amino acids 21 and 22 of the N-terminus of the Aα chain and amino acids 394 and 395 from the N-terminus of the γ-chain.[13] Several of the low molecular weight products generated during fibrinogen proteolysis were shown to associate with β_2-integrin receptors modulating subsequent adhesive interactions.[39] The ability of this membrane protease to degrade fibrin clots led to the suggestion that neutrophil mediated fibrinolysis is not dependent on degranulation.[14] The low molecular weight *fibrin* peptides generated during this process were shown to inhibit thrombin induced platelet aggregation, whilst the higher molecular weight products could partially overcome platelet induced resistance of fibrin to plasminolysis.[40] In addition to its effects on fibrinogen, this protease was also shown to degrade C-reactive protein (CRP)[38] generating several biologically active peptides capable of modulating a number of important pro-inflammatory neutrophil responses.[41-44] This novel membrane associated protease thus appeared to be associated with a number of processes influencing both the inflammatory and coagulation pathways (see figure 1).

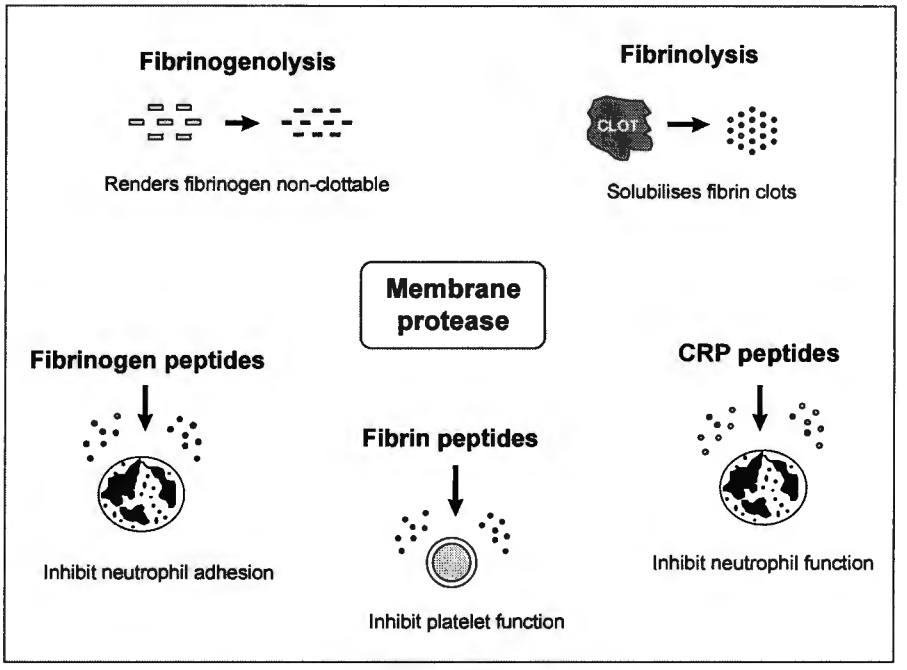


Figure 1: *The 600 kDa membrane associated protease has been reported to have numerous biological functions which result from its proteolysis of fibrinogen, fibrin and C-reactive protein.*

Whilst this protease had been partially characterised with respect to its molecular weight, its inhibitor profile and its pattern of substrate degradation,[13,14,38] it was yet to be purified from crude extracts. Thus many of its structural and catalytic properties remained to be elucidated.

The present study aimed to purify and further characterise the 600 kDa membrane associated protease. In addition this study sought to confirm the association of neutrophil derived fibrinogen degradation products with β_2-integrin receptors on the neutrophil surface.

References

1. Borregaard N. (1988) *Eur. J. Haematol.* **41**, 401.
2. Cohen MS. (1994) Clin. Infect. Dis. **18** (Suppl. 2), S170.
3. Levy O. (1996) *Eur. J. Haematol.* **56**, 263.
4. Henry RL. (1965) *Thromb. Diath. Haemorrh.* **13**, 35.
5. Barnhardt MI (1965) *Fed. Proc.* **24**, 846.
6. Pool JC. (1964) *Sci. Basis Med. Annu. Rev.* 55.
7. Gillis S, Furie BC, Furie B. (1997) *Semin. Haematol.* **34**, 336.
8. Heiden M, Seitz R, Egbring R. (1996) *Semin. Thromb. Haemostas.* **22**, 497.
9. Plow EF, Edgington TS. (1975) *J. Clin. Invest.* **56**, 30.
10. Gramse M, Bingenheimer C, Schmidt W, Egbring R, Havemann K. (1978) *J. Clin. Invest.* **61**, 1027.
11. Bilezikian SB, Nossel HL. (1977) *Blood.* **50**, 21.
12. Bach-Gansmo ET, Halvorsen S, Godal HC, Skjonsberg OH. (1994) *Thromb. Res.* **73**, 61.
13. Kelly SL, Adams SA, Robson SC, Kirsch RE, Shephard EG. (1994) *Biochem. J.* **298**, 689.
14. Adams SA, Kelly SL, Kirsch RE, Robson SC, Shephard EG. (1995) *Blood Coag. Fibrinol.* 6, 693.
15. Adams SA, Kelly SL, Kirsch RE, Shephard EG. (1998) *Blood Coag. Fibrinol.* 9, 307.
16. Succhard SJ, Boxer LA (1994) *J. Immunol.* **152**, 290.
17. Berton G, Fumagalli L, Laudanna C, Sorio C. (1994) *J. Cell Biol.* **126**, 1111.
18. Kazura JW, Wenger JD, Salata RA, Budzynski AZ, Goldsmith GH. (1989) *J. Clin. Invest.* **83**, 1916.
19. Fischer EG, Vogel P, Ziegler AS, Kirkpatrick CJ. (1991) *Haemostasis.* **21**, 141.
20. Gross TJ, Leavell KJ, Peterson MW. (1997) *Thromb. Haemostas.* **77**, 894.
21. Leavell KJ, Peterson MW, Gross TJ. (1996) *Am. J. Resp. Cell. Mol. Biol.* **14**, 53.
22. Carson SD, Brozna JP. (1992) *Blood Coag. Fibrinol.* **4**, 281.

23. Edwards RL, Rickles FR. (1992) *Semin. Haematol.* **29**, 202.
24. Bailey K, Bettelheim FR, Lorand L, Middlebrook WR. (1951) *Nature*. **167**, 233.
25. Gray AJ, Bishop JE, Reeves JT, Mecham RP, Laurent GJ. (1995) *Am. J. Cell. Mol. Biol.* **12**, 684.
26. Rulot H. (1904) *Arch. Int. Physiol. Biochim.* **1**, 152.
27. Opie EL. (1907) J. Exp. Med. **9**, 391.
28. Plow EF. (1980) *Biochim. Biophys. Acta.* **630**, 47.
29. Gramse M, Bingenheimer C, Havemann K. (1980) *Thromb. Res.*. **19**, 201.
30. Pakianathan DR. (1995) *J. Leuk. Biol.* **57**, 699.
31. Wright SD, Weitz JI, Huang AJ, Levin SM, Silverstein SC, Loike JD. (1988) *Proc. Natl. Acad. Sci. USA*. **85**, 7734.
32. Loike JD, Sodeik B, Cao L, Leucona S, Weitz JI, Detmers PA, Wright SD, Silverstein SC. (1991) *Proc. Natl. Acad. Sci. USA*. **88**, 1044.
33. Gresham HD, Adams SP, Brown EJ. (1992) *J. Biol. Chem.* **267**, 13895.
34. Zhou M, Brown EJ. (1993) *J. Exp. Med.* **178**, 1165.
35. Altieri D, Plescia J, Plow EF. (1993) *J. Biol. Chem.* **268**, 1847.
36. Nathan C, Srimal S, Farber C, Sanchez E, Kabbash L, Asch A, Gailit J, Wright SD. (1989) *J. Cell Biol.* **109**, 1341.
37. Senior RM, Skorgen WF, Griffin GL, Wilner GD. (1986) *J. Clin. Invest.* **77**, 1014.
38. Shephard EG, Kelly SL, Anderson R, Fridkin M. (1992) *Clin. Exp. Immunol.* **87**, 509.
39. Kelly SL. (1995) A neutral protease of the neutrophil surface: its role in the proteolysis of C-reactive protein and fibrinogen - PhD Thesis.
40. Adams SA. (1996) Proteases of the neutrophil membrane represent an alternative fibrinolytic pathway to that mediated by plasmin. (PhD Thesis, University of Cape Town).
41. Shephard EG, De Beer SM, Anderson R, Strachan AF, Nel AE, De Beer FC. (1989) *J. Immunol.* **143**, 2974.
42. Shephard EG, Anderson RA, , Rosen O, Myer MS, Fridkin M, Strachan AF, De Beer FC. (1990) *J. Immunol.* **145**, 1469.
43. Zhong W, Zen Q, Tebo J, Schlottmann K, Coggeshall M, Mortensen RF. (1998) *J. Immunol.* **161**, 2533.
44. Jones SA, Novick D, Horiuchi S, Yamamoto N, Szalai AJ, Fuller GM. (1999) *J. Exp. Med.* **189**, 599.

Chapter Two

The Neutrophil

Neutrophils represent the first line of defence against microbial pathogens and are important mediators of the inflammatory response. They are the most prevalent of the circulating leukocytes, comprising approximately 55-60% of white cells in peripheral blood; lymphocytes, monocytes, basophils and eosinophils make up the remainder (figure 1).[1] The latter two groups, together with the neutrophils constitute a population of leukocytes known as granulocytes - so named because of the abundance of granules in their cytoplasm - of which neutrophils comprise over 90%.[1]

As a phagocytic cell the phylogeny of the neutrophil long precedes the emergence of the lymphocyte, suggesting its fundamental importance in host defence.[2] To fulfil its role as a phagocyte, the neutrophil is equipped with an armoury of antimicrobial machinery which enables it to migrate to sites of inflammation, engulf invading micro-organisms and liberate an array of toxic oxygen metabolites and potent proteases aimed at the destruction of internalised material.[3-6] Despite the long held perception of the neutrophil as a purely mechanical phagocytic cell, it is rapidly emerging as a highly sophisticated inflammatory cell and an important regulator of the acute inflammatory response.

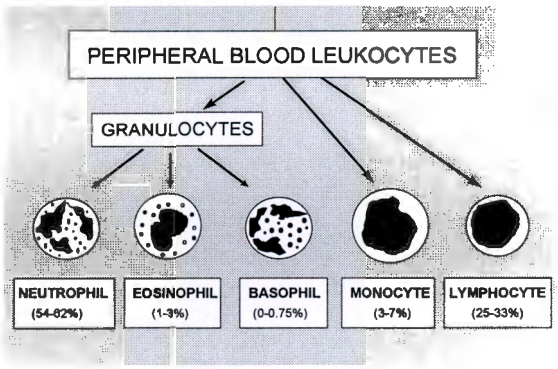


***Figure 1**: Peripheral blood leukocytes. The granulocyte population consists of neutrophils, eosinophils and basophils. The proportion of each group in the peripheral blood is indicated.*

Neutrophil maturation

Neutrophils evolve from pluripotent stem cells.[7] The myeloblast is the first recognisable precursor cell and, following the acquisition of azurophil granules, it evolves into the promyelocyte. The promyelocyte divides to form the myelocyte, in which the specific granules are synthesised.[8] During the final stages of maturation there is no cell division and the cell passes through the metamyelocyte stage and on to the band cell with a sausage shaped nucleus. As the band cell matures the nucleus assumes a lobulated configuration.[1]

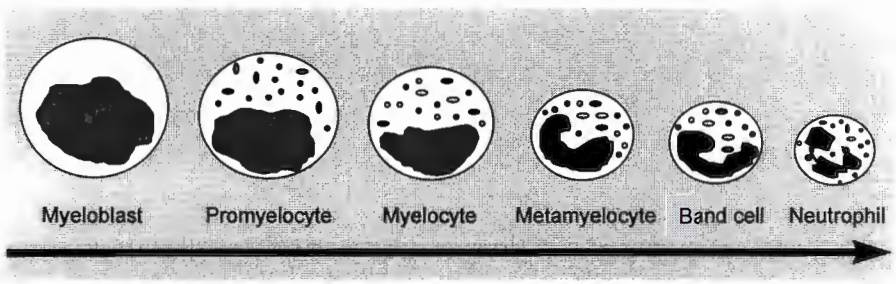


Figure 2: *The neutrophil precursors. Progression towards maturity is characterised by he progressive acquisition of the various granule subsets.*

Neutrophils are produced in the bone marrow at a rate of approximately 70 million/minute.[9] After maturation, the neutrophil usually spends up to 5 days within the bone marrow prior to release in to the circulation where it has a lifespan of approximately 2-3 days.[6,9] Under normal conditions, about 90% of the neutrophil pool is in the bone marrow, 2-3% in the circulation and the rest in the tissues.[1]

An overview of neutrophil function

In performing its phagocytic function, the neutrophil is involved in a series of events, starting with its migration into affected tissues and culminating in the internalisation and destruction of foreign particulate

matter (see figure 3). This process is broadly divided into five steps each of which is reviewed briefly below.

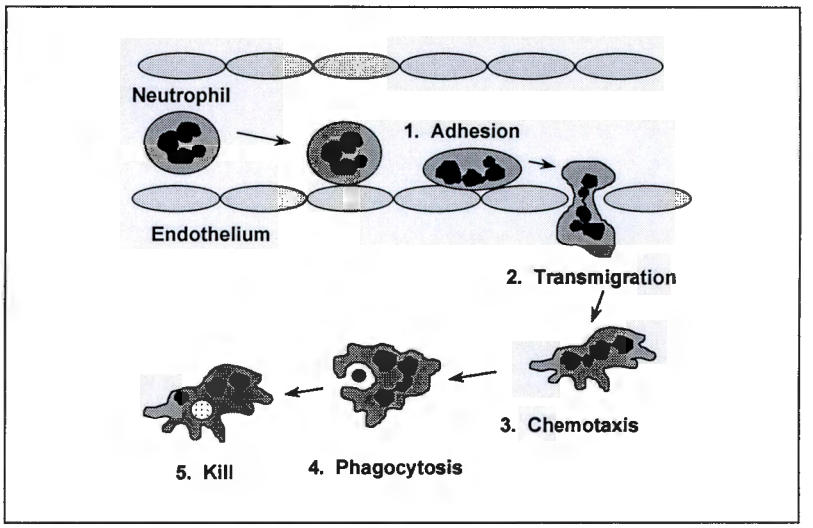


Figure 3: *A schematic representation of the five basic steps involved in the neutrophil response to inflammation, beginning with migration from the vasculature and culminating in destruction of ingested material.*

1. Adherence

The first step in the acute inflammatory response is the attachment of the neutrophil to the endothelial cell (EC) surface.[10] Tissue injury results in the release of a variety of inflammatory mediators which are capable of promoting neutrophil adhesion to EC by enhancing the avidity of neutrophil surface adhesion molecules for EC ligands. At the same time, inflammatory mediators also induce the expression EC adhesion molecules which further support neutrophil adhesion.[11] The molecular

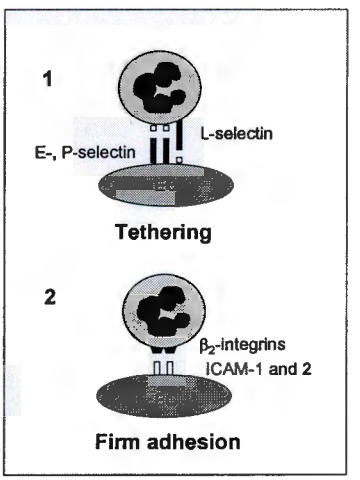


Figure 4: *Molecules involved in neutrophil-EC interactions*

basis of neutrophil-EC adhesion is discussed in detail in a later section, and is therefore eluded to only briefly here. The initial interaction of the neutrophil with the endothelium, known as tethering, is mediated by a family of adhesion molecules known as selectins which are found both on the EC (E- and P-selectin) and on the neutrophil (L-selectin) (Figure 4). Tethering precedes firm adhesion which results in arrest of the cell and is mediated by interactions between β_2-integrins (CD11/CD18 family) and EC counter-receptors, including intercellular adhesion molecules 1 and 2 (ICAM 1 and 2).[11]

2. Transmigration

After strong adhesion to ECs, neutrophils migrate into tissues under the influence of promigratory factors (figure 5). Many of the cytokines that trigger strong adhesion may also act as chemotactic factors, e.g. interleukin 8 (IL-8). The increased vascular permeability at sites of inflammation allows the migration of neutrophils from the vasculature. It has been suggested that the transience of both P-selectin expression and β_2-integrin mediated adhesion provides the reversibility required for the migration of neutrophils across ECs.[11]

3. Chemotaxis

Once the neutrophil has traversed the endothelium it must migrate to the site of inflammation. It does so by the process of chemotaxis which involves directed movement across a concentration gradient of chemoattractant molecules (figure 5).[5] A variety of chemotactic agents are involved in neutrophil migration towards the inflammatory site and these may be derived from the plasma (e.g. C5a, kallikrein, fibrinopeptide B), from inflammatory cells (e.g IL-8,

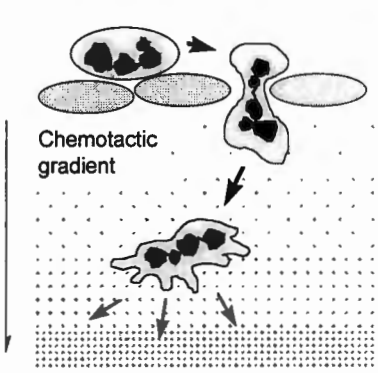


Figure 5: *Directed migration of neutrophils across a chemotactic gradient allows for their accumulation at inflammatory sites.*

leukotriene B_4, platelet activating factor) or micro-organisms (e.g. FMLP [N-formyl-methionyl-leucyl-phenylalanine]).[12] Chemotaxis is initiated by the binding of chemoattractants to neutrophil surface receptors, with the subsequent activation of signal transduction events which induce dramatic rearrangements of the cytoskeleton, facilitating directed migration.[13,14]

4. Phagocytosis

Having arrived at the inflammatory focus, neutrophils ingest foreign particulate matter or cellular debris by a process known as phagocytosis.[6,13] This process can be divided into three distinct steps (figure 6):

(i) The first involves the attachment of the particle to the cell surface. This process is facilitated by the coating of particles with serum opsonins, most commonly complement fragments or IgG, which are recognised by receptors on the cell surface (IgG by IgG-Fc receptors, and C3b and C3bi by complement (C3) receptors CR1 and CR3 respectively).[6]

(ii) Binding is followed by particle engulfment which is characterised by a flow redistribution of the plasma membrane to surround the particle with finger-like protrusions known as pseudopodia. Fusion of the pseudopodia around the particle yields a membrane bound organelle of plasma membrane origin.[15]

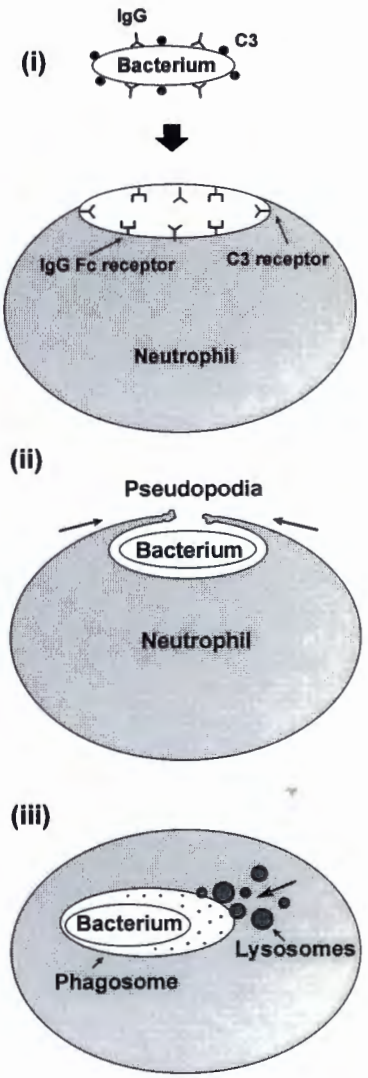


Figure 6: *The 3 steps of phagocytosis*

(iii) In the final step of the process, phagosomes are transformed into functional phagolysosomes through the aquisition of acid hydrolases and ion pumps (required for the acidification of the endosomal compartment) via fusion with lysosomes.[16,17]

5. Neutrophil killing

The ingestion of foreign particles sets into motion the potent microbicidal machinery of the neutrophil. Concomitant with phagocytosis there is a burst of oxygen consumption and activation of the hexose monophosphate shunt which generates NADPH (figure 7).[6] NADPH oxidase is assembled on the phagosomal (and plasma) membranes, into an active complex together with flavoproteins and cytochrome b. This complex generates reactive oxygen intermediates such as superoxide anion (O_2^-) through the oxidation of NADPH (figure 7).[6] Superoxide anion is converted to hydrogen peroxide (H_2O_2) either by spontaneous dismutation or through the action of superoxide dismutase.[5] Myeloperoxidase, liberated from the azurophil granules, catalyses the conversion of H_2O_2 to toxic intermediates such as hypochlorous acid (HOCl). HOCl is an extremely powerful oxidant with potent microbicidal potential due to its ability to oxidise and halogenate micro-organisms.[18]

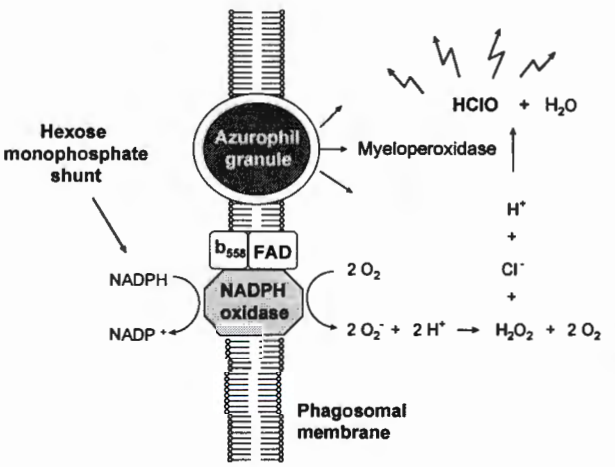


Figure 7: *A schematic representation of the metabolic events related to the neutrophil respiratory burst. The cytochrome b_{558}-FAD-NADPH oxidase system is assembled on the membrane of the phagolysosome. This facilitates the generation of oxygen radicals which are converted to the toxic hypochlorous acid by myeloperoxidase released from azurophil granules.*

Acidification of the phagolysosome assists in neutrophil destruction of internalised micro-organisms. Some microbes are killed by a low pH, and a number of neutrophilic antimicrobial systems function best at low pH.[5] Acidification occurs via the acquisition, from the lysosomes, of acid hydrolases, as well as the appropriate ion pumps and channels required for accumulation of hydrogen ions within the phagocytic compartment.[5]

Neutrophil granules contain a vast store of bactericidal proteins and peptides, including bacterial permeability increasing protein (BPI), defensins, azurocidin, and many others, which can destroy ingested microrganisms.[3] Neutrophil granules also contain a number of potent proteases, including elastase, cathepsin G and proteinase 3, which contribute both toward bactericidal activity and digestion of microbial debris.[19]

Neutrophil granules

Neutrophil granules are intimately involved in both neutrophil *proteolytic* as well as *adhesive* processes, the two subjects of the experimental section of this thesis. A considerable portion of this review will therefore be devoted to this very important group of organelles.

Over the last 15 years the perception of neutrophil granules has changed dramatically. Long viewed as simple bags of proteolytic or bactericidal proteins destined for release into the phagosome or the extracellular millieu, neutrophil granules have become increasingly recognised as the essential machinery which enables the neutrophil to adapt rapidly, and fundamentally, to changes in its environment. The incorporation of granule proteins into the external membrane of the neutrophil following fusion of granules with the plasma membrane allows the neutrophil to rapidly change the profile of proteins expressed on its surface.[20,21] Depending on the granule population released, neutrophils may undergo upregulation of specific functions such as adherence, migration, phagocytosis or respiratory burst.[22-24]

The neutrophil granules have emerged as an extremely heterogeneous group of organelles. The simplest classification includes four groups of neutrophil granules distinguished on the basis of their protein content, size and density (figure 8). These include the azurophil (primary), specific (secondary) and gelatinase (tertiary) granules as well as a recently identified group endocytic origin known as secretory vesicles.[2]

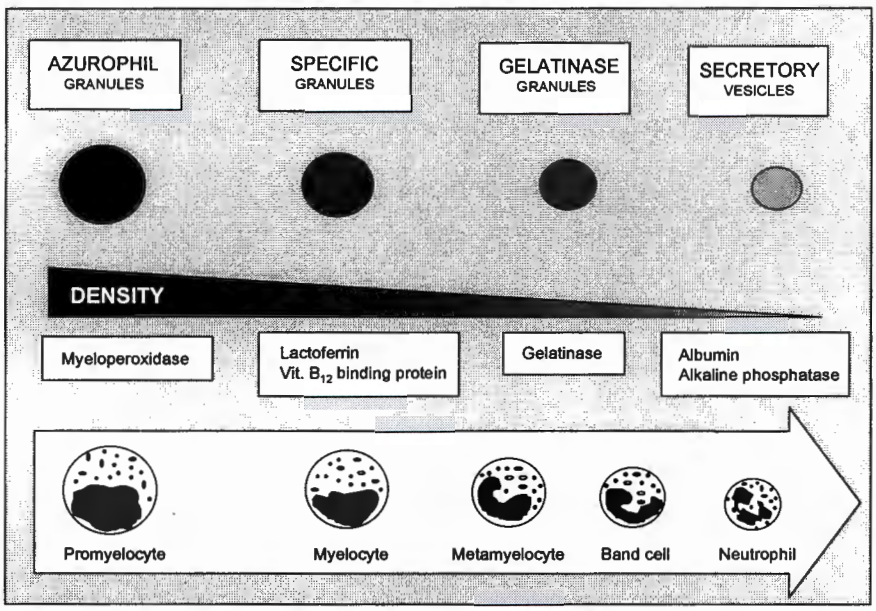


Figure 8: *Neutrophil granules differ in their size, density and protein content reflecting the stage of neutrophil maturation at which they are formed.*

Following synthesis of granule proteins, the packaging of granules in the Golgi apparatus involves condensation of protein within cysternae and release of Golgi derived coated vesicles which ultimately lose their coats and fuse to form immature granules. Further condensation occurs during maturation of granules.[8,25] In view of the considerable heterogeneity of neutrophil granules, it would appear that sorting of the various proteins into different granules requires a highly sophisticated mechanism. Such a mechanism is not in fact required, as all granule proteins that are synthesised at a particular point in time are packaged into the same granules. Thus the basis for granule heterogeneity lies in the

differences in gene expression and protein synthesis associated with the different stages of the neutrophil maturation cycle.[26,27] Indeed, it has long been known that the different neutrophil granules arise at specific stages of neutrophil development. Azurophil (primary) granules are the first to appear in the cytoplasm during the neutrophil maturation process, at the promyelocyte stage. Next follow the specific (secondary) granules which are formed at the myelocyte/metamyelocyte stage and this is accompanied by a cessation in the production of azurophil granules.[8] This is followed by the gelatinase (tertiary) granules which develop at the metamyelocyte/band cell stage.[28] The secretory vesicles are the last to arise in the cytoplasm and first appear in band cells and segmented cells.[2]

The various granule subsets show marked differences in their propensity for mobilisation following neutrophil activation. The order of mobilisation correlates negatively with the size and density of the granules. Secretory vesicles represent the most easily mobilisable set of granules followed by the gelatinase, specific and lastly azurophil granules.[29-31] In addition to membrane constituents, neutrophil granules also contain a variety of proteins and peptides of major importance within their interiors. These include a number of proteases and antimicrobial proteins and peptides involved in neutrophil migration and destruction of invading micro-organisms.[2,3] Because the different categories of neutrophil granule reflect differences in gene expression and synthesis of granule proteins over time, rather than post-synthesis sorting mechanisms, there is considerable overlap between the various groups with respect to granule contents.[27] Thus, rather than comprising completely separate groups, neutrophil granules form a continuum from azurophil through to gelatinase granules, with a number of proteins being shared between the groups.[32,33] However, for practical purposes, granules can be divided into four major categories, each of which is reviewed separately below.

1. Azurophil granules

Azurophil granules are the stores of most proteolytic and bactericidal proteins. Being the first to appear during neutrophil maturation they are also known as primary granules. Azurophil granules were initially distinguished from other granules on the basis of their affinity for the basic dye azure A (which stains acid mucopolysaccharide),[34] and later on the basis of their myeloperoxidase content which is not present in other granules.[8,35] Azurophil granules are generally regarded as being spherical and oval in contrast to specific granules which tend to be more elongated and irregular.[8] The azurophil granules contain myeloperoxidase,[36] the serine proteases elastase,[37] cathepsin G,[38] and proteinase 3,[39] acid hydrolases,[40] and a variety of microbicidal proteins including lysozyme,[41] defensins,[42] azurocidin,[43] and bactericidal/permeability increasing protein.[44] Until recently, azurophil granules have generally been considered to be analogous to lysosomes, since they are membrane bound organelles that contain acid hydrolases that have not yet entered into a digestive event.[40,41,45] However, recent studies have revealed that the classic lysosomal membrane markers, lysosome associated membrane protein (LAMP) 1 and 2, are not present in the membranes of azurophil granules.[46] This has led to the suggestion that these granules do not in fact represent true lysosomes, but behave more like regulated storage vesicles which are mobilised to the phagosome during ingestion of micro-organisms.[47] The *membranes* of azurophil granules do not contain any receptors or other functional proteins involved in the

Azurophil granule constituents*

Membrane:

- CD63
- CD68

Matrix:

- Human neutrophil elastase
- Cathepsin G
- Proteinase 3
- Myeloperoxidase
- Azurocidin
- β-Glucuronidase
- Lysozyme
- Acid β -glycerophosphatase
- Acid mucopolysaccharide
- α_1-antitrypsin
- Defensins
- α-mannosidase
- Bactericidal permeability increasing protein
- Heparin binding protein
- Sialidase
- Ubiquitin protein

* Tables on pages 16-19 are adapted from reference 25

interaction of neutrophils with their environment.[2] Although CD63 and CD68 have been identified on azurophil granule membranes, their significance, if any, is unclear.[48,49] Of all the granule subsets, azurophil granules have the lowest propensity for mobilisation.[31] It has thus been suggested these granules may play less of a role extracellularly than other classes of neutrophil granules,[31] and that their major role may only be executed after disintegration of the cell.[2] However, secretion of azurophil granules does occur to some extent,[50] and a number of recent studies have demonstrated the presence of elastase and cathepsin G bound to the cell surface of activated neutrophils.[51-54] Moreover, neutrophils have also been shown to express the azurophil granule microbicidal factor BPI on their surfaces.[55] It thus appears that regulated release of azurophil granules, albeit it at relatively low levels, may allow the constituents of these granules to perform important functions extracellularly.

2. Specific granules

The specific granules are the second subset of neutrophil granules to develop during neutrophil maturation and hence are referred to as secondary granules. Specific granules tend to be more irregular and elongated in shape.[8] These granules are distinguished by their content of lactoferrin and vitamin B_{12}-binding protein, which are unique to this subset of organelles.[56] In contrast to azurophil granules, the *membranes* of specific granules serve as important reservoirs of receptors and other proteins and, following their translocation to the neutrophil membrane, these constituents boost the capacity of

Specific granule constituents*

Membrane:

- Cytochrome b_{558}
- Mac 1 (CD11b)
- Laminin receptor
- Fibronectin receptor
- Vitronectin receptor
- Thrombospondin receptor
- FMLP-receptor
- Tumour necrosis factor receptor
- G-protein α-subunit
- Urokinase plasminogen activator receptor

Matrix:

- Lactoferrin
- Vitamin B_{12} binding protein
- Collagenase
- Lysozyme
- Urokinase plasminogen activator
- Gelatinase
- β_2-microglobulin
- Histaminase
- Heparanase

the neutrophil to perform various functions. Since these granules represent the major store of flavocytochrome b_{558}, an essential component of NADPH, they contribute significantly towards the respiratory burst.[57,58] In addition they contain a variety of adhesion receptors (including Mac-1,[59] and the laminin-, fibronectin- and vitronectin receptors[23]) which add to the contributions made by the more easily mobilisable gelatinase and secretory granules (see later). Specific granules are also important reservoirs of receptors for FMLP[60,61] and tumour necrosis factor (TNF).[62]

With respect to *matrix* proteins, specific granules are dominated by lactoferrin,[2] whose precise function remains unknown, although it has been reported to possess microbicidal activity.[3] Another constituent of the matrix worth mentioning is collagenase,[63] due to its potential to facilitate neutrophil migration through tissues,[64] and to cause tissue destruction when uncontrolled.[18] The matrix also contains a variety of other proteins including lysozyme,[41] urokinase plasminogen activator (u-PA),[65] gelatinase,[28] and vitamin B_{12} binding proteins.[66]

3. Gelatinase granules

Gelatinase (tertiary) granules are the third subset of neutrophil granules to develop during neutrophil maturation, with maximal synthesis occurring during metamyelocyte/band cell stage.[28] Like specific granules, gelatinase granules are peroxidase negative but differ from the former in that they lack lactoferrin.[32] These granules, which are lighter and smaller than specific granules, store the bulk of neutrophil gelatinase, although

Gelatinase granule constituents*

Membrane:

- Mac 1 (CD11b)
- Cytochrome b_{558}
- FMLP-receptor
- Urokinase plasminogen activator receptor
- Diacylglycerol-deacylating enzyme

Matrix:

- Gelatinase
- Lysozyme
- β_2-microglobulin
- Acetyltransferase

some gelatinase is also stored in the specific granules.[32] Gelatinase granules are mobilised more readily than specific granules in response to inflammatory mediators.[29,31,67] Although the membrane of gelatinase granules appears to be similar to that of specific granules,[68] mobilisation of gelatinase granules during neutrophil activation does not make a major contribution to the neutrophil membrane because these granules are small and relatively few.[32] The significance of mobilisation of gelatinase granules, therefore, does not appear to be their contribution to membrane proteins, but rather the release of gelatinase, which due to the highly concentrated state in which it is stored, occurs in substantial amounts.[2] As type IV collagen (of basement membranes) and type V collagen (of interstitial tissues) are major substrates of gelatinase,[69-71] it is likely that exocytosis of gelatinase granules plays an important role in the migration of neutrophils through basement membranes and into the underlying tissue.[72,73]

4. Secretory vesicles

The observation that a variety of receptors, including Mac-1, can become incorporated into the plasma membrane without the associated exocytosis of cytoplasmic granules, led to a search for an alternative mobilisable source of these receptors. This resulted in the discovery of the secretory vesicle, the most rapidly mobilisable intracellular structure in neutrophils.[21,74-76] The membranes of secretory vesicles are particularly rich in a variety of important receptors and proteins, including Mac-1,[77] the FMLP receptor,[78] the u-PA receptor,[79] as well as cytochrome b_{558},[77] and CR1.[80] The only known intravesicular constituent of secretory vesicles is plasma; thus albumin currently serves as a marker of these vesicles.[81] This implies that secretory vesicles are

Secretory vesicle constituents*

Membrane:

- Alkaline phosphatase
- Mac 1 (CD11b)
- Cytochrome b_{558}
- CR1
- FMLP-receptor
- Urokinase plasminogen activator receptor

Matrix:

- Plasma proteins

endocytic in origin. Secretory vesicles are, however, distinct from the endocytic vesicles of constitutive pinocytosis and, once mobilised, are not reassembled.[81-83]

The extreme sensitivity with which the neutrophil responds to inflammatory stimuli by mobilising secretory vesicles suggests that these vesicles play a primary role in the initiation of the neutrophil inflammatory response. The secretory vesicles are thought to be mobilised when the neutrophil establishes primary rolling contact with the endothelium. The mobilisation of these vesicles may be mediated by signalling through selectins,[84,85] or by inflammatory mediators liberated from the endothelium.[86,87] Incorporation of these vesicles into the plasma membrane endows the neutrophil with a variety of receptors (in particular β_2-integrins and FMLP receptors) which render the neutrophil highly responsive to inflammatory stimuli and primed for migration into tissues.[88-90]

Thus, the heterogeneity of the neutrophil cytoplasmic granules, together with their differential rates of mobilisation, provides the neutrophil with the ability to rapidly adapt to changes in its environment in a highly regulated manner. This is summarised below with the aid of figure 9. Whilst mobilisation of the secretory vesicles appears to initiate the increased neutrophil adhesiveness required for attachment to the endothelium, subsequent release of gelatinase granules facilitates neutrophil migration through tissues through the ability of gelatinase to degrade collagen. The specific granules are next to be released with the subsequent translocation cytochrome b_{558} to the neutrophil membrane, which is vital for the assembly and activation of the NADPH-oxidase-cytochrome b_{558} complex and the subsequent respiratory burst. These granules also bolster the supply of various adhesion receptors important in phagocytosis. Finally, following internalisation of the foreign particle, azurophil granules release a variety of antimicrobial proteins and proteases into the phagolysosome which destroy internalised particles.

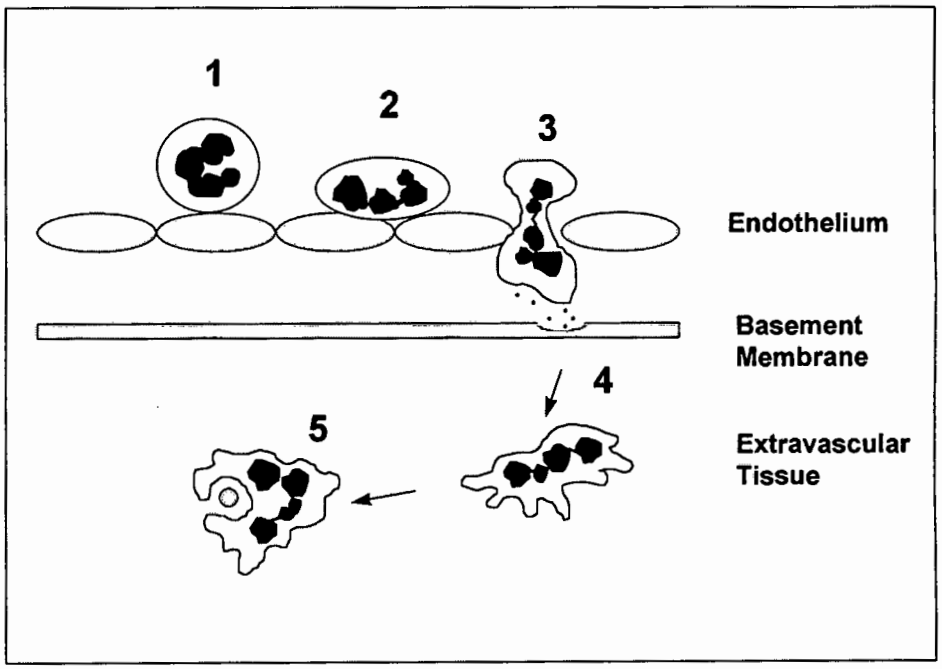


Figure 9: *The role of cytoplasmic granules in the neutrophil inflammatory response. (1) Circulating neutrophils become tethered to activated endothelium primarily via selectins, both on the neutrophil and the endothelium. Ligation of L-selectin may signal for the release of secretory vesicles. (2) The incorporation of secretory vesicles into the plasma membrane increases the surface expression of CD11b/CD18 (Mac-1) enhancing the potential for firm adhesion to the endothelium. (3) Exocytosis of gelatinase from gelatinase granules as well as elastase from azurophil granules may facilitate the degradation of the basement membrane and thus assist in transmigration into the extravascular space. (4) The expression of receptors for chemotactic molecules such as FMLP (released from secretory vesicles and specific and gelatinase granules facilitates directed migration towards the inflammatory focus. (5) Mobilisation of specific granules to the neutrophil surface may enhance the phagocytic potential of the neutrophil via the increased expression of Mac-1 on the plasma membrane. Fusion of specific and azurophil granules with the phagosome supplies cytochrome b_{558} and myeloperoxidase respectively, which allow for the generation of toxic oxygen metabolites. The release of bactericidal agents and potent digestive proteases facilitates the intracellular digestion of internalised particles.*

Neutrophil proteases

Proteases of the neutrophil granule

I. Serine proteases

Human neutrophil elastase (HNE), cathepsin G, proteinase 3 and azurocidin belong to a family of multifunctional haematopoietic serine proteases, often referred to as serprocidins. In addition to their broad spectrum proteolytic activity, these powerful proteases are also endowed with microbicidal properties.[19,91] The serprocidins are structurally related cationic glycoproteins of similar size (25-29 kDa).[19] Within this group, azurocidin is exceptional in that it is a sterile enzyme, lacking proteolytic activity due to mutations in 2 out of 3 residues in the presumed catalytic triad of the protein.[92,93] Multiple and sometimes overlapping functions are a characteristic of this family of proteases.[19] They exhibit a broad spectrum of proteolytic activity and have been shown to modulate a wide variety of important cellular processes (see below). Their microbicidal activity may involve both proteolytic and non-proteolytic mechanisms.[3,19] The serprocidins are broadly cytotoxic to Gram-negative and positive bacteria, fungi, protozoa as well as mammalian cells.[3] They may act alone or in combination with other antimicrobial agents. They may play an additional role in host defences by converting cytotoxins from active to inactive forms.[19]

The serine proteases of the azurophil granule are structural relatives of the granzymes of the granules of cytotoxic T cells, as well as certain mast cell proteases (see figure 10).[94] The genes of HNE, proteinase 3 and azurocidin are organised in a single locus on chromosome 19,[95] whilst the gene for cathepsin G is located, together with those for two cathepsin G-like proteases of cytotoxic T-lymphocytes, in a single cluster on chromosome 14.[96] There is strong evidence to suggest that these proteases have arisen from a single ancestral gene via repeated gene duplications and mutations.[97]

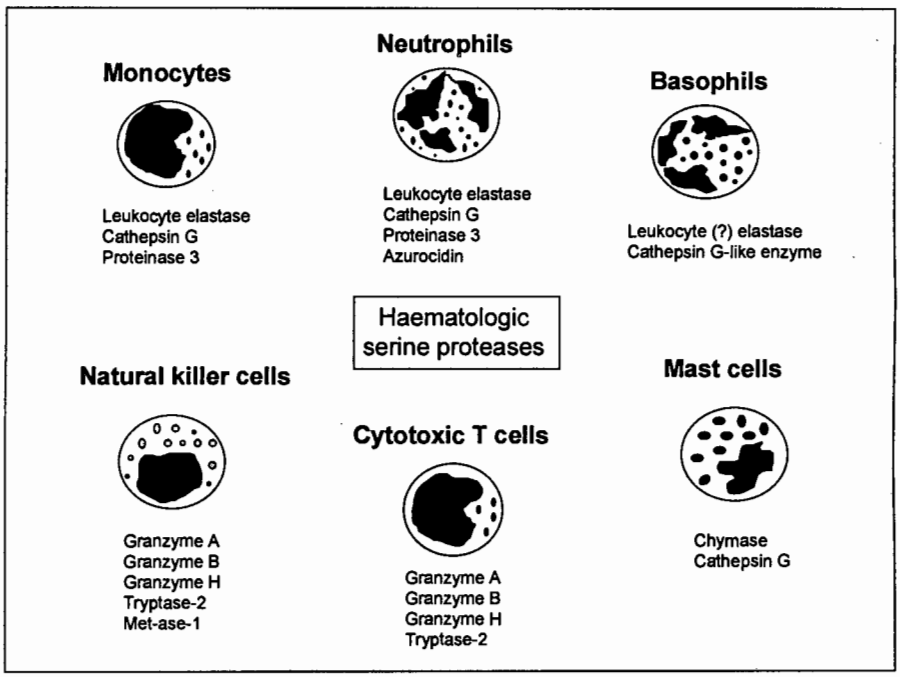


Figure 10: *The haematopoietic serine proteases are a closely related family of proteases stored in the cytoplasmic granules of a variety of haematopoietic cells.*

The synthesis of the serprocidins occurs during the promyelocyte stage of neutrophil maturation.[98] Co-translational removal of a signal peptide yields a zymogen containing a 2-amino-acid propeptide aminoterminally, which prevents premature activation of the protease during intracellular transport.[56] Subsequent removal of the prodomain in the pregranular or granular compartments yields the mature and active enzyme.[98] Each member of this family is discussed in turn below.

1. Human neutrophil elastase

Human neutrophil elastase (HNE) is a potent, broad-specificity serine protease of considerable (patho)physiological importance. When appropriately controlled, its proteolytic activity regulates a variety of important biological processes; however, when inappropriate or prolonged, HNE mediated proteolysis may result in extensive tissue

damage and a variety of disabling and destructive diseases including emphysema, glomerulonephritis and rheumatoid arthritis.[99-103]

HNE is a single chain, cationic 30 kDa glycoprotein, 218 amino acids in length.[104] As with other serine proteases, HNE activity is dependent on the catalytic triad His_{41}-Asp_{88}-Ser_{173} centred at the HNE reactive site.[104,105] When a substrate is introduced into the active site pocket, the transfer of a proton within the triad converts the Ser_{173} into a highly reactive nucleophile which is capable of disrupting the peptide bond within the target substrate.[105] HNE is classified as an elastase because it belongs to a small group of proteases which can degrade insoluble elastin, a highly crosslinked rubber-like macromolecule which provides structural integrity and flexibility to connective tissue matrices.[106] However, despite the specificity of its name, neutrophil elastase can also destroy most other matrix proteins (including fibronectin, fibrinogen, collagen types III, IV and VI, and proteoglycans) as well as coagulation factors, immunoglobulins and complement components.[53,107,108]

The ability of HNE to degrade extracellular matrix proteins has led to the suggestion that it may play an important role in neutrophil migration, both from the vasculature and through the tissues, by digesting proteins in the cell's path.[18] Indeed, recent evidence indicates that neutrophil migration across confluent EC monolayers *in vitro* is HNE dependent.[109] This is supported by *in vivo* observations that leukocyte emigration across venular endothelium into interstitium, during experimentally induced inflammation, is markedly diminished in the presence of HNE inhibitors.[110-112] Moreover, the recent finding that HNE binds specifically to the adhesion receptor Mac-1, and is essential for reversal of integrin mediated attachment of neutrophils to the extracellular matrix, has suggested an additional mechanism whereby this protease may promote neutrophil emigration.[113] HNE also has also been shown to upregulate the expression of the β-subunit of the CD11/CD18 integrin complex and, to a lesser extent, CD11b on the neutrophil surface.[111] This upregulation has been shown to promote neutrophil migration but not adhesion at inflammatory sites, although the mechanism for this remains unclear.[111]

HNE is a multifunctional protease which is involved in a variety of biological processes beyond its role in connective tissue proteolysis (see figure 11).

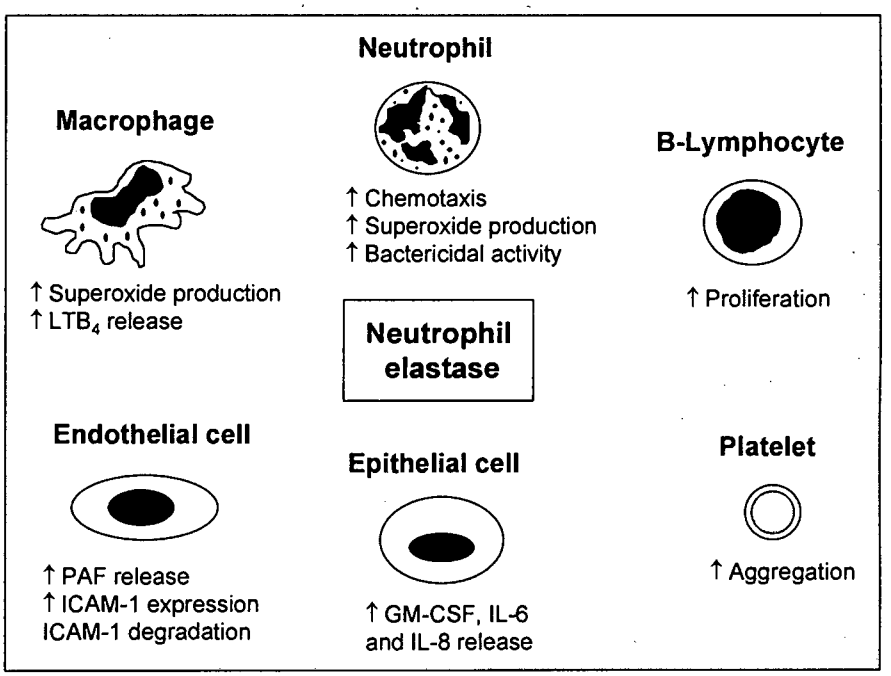


Figure 11: *A schematic representation of the multiple effects that human neutrophil elastase may have on the function of a variety of cell types. In addition HNE may have a variety of indirect effects as a result of proteolysis of a variety of plasma proteins.*

HNE mediated proteolysis can influence *neutrophil* function in several ways. It can promote the influx of neutrophils into sites of inflammation by inducing release of granulocyte macrophage colony stimulation factor (GM-CSF), IL-6 and IL-8 from epithelial cells,[114-116] by cleaving α_1-antiproteinase inhibitor to generate a chemotactic fragment,[117] and by stimulating the release of leukotriene B_4 (LTB_4) from mononuclear phagocytes.[118] HNE has also been shown to augment superoxide generation in response to various stimuli, and this may significantly enhance neutrophil microbicidal activity, as well as the potential for host tissue injury.[119] HNE has been shown to exhibit bactericidal activity *in vitro*,[120,121] which is not necessarily dependent on its proteolytic activity.[121] The physiological

significance of these *in vitro* observations has recently been confirmed by *in vivo* studies, using mice rendered HNE deficient by targeted mutagenesis of the HNE gene.[122] Mice deficient in HNE showed markedly higher morbidity and mortality rates following intraperitoneal inoculation with Gram negative bacteria (klebsiella pneumonia and escherichia coli) but not Gram positive bacteria (staphylococcus aureus) than normal mice. This appeared to be due to impaired intraphagosomal killing of ingested Gram negative bacteria. [122]

HNE has also been shown to modulate the function of a variety of other cell types. It is known to prime macrophage superoxide production,[123] modify platelet aggregation,[124] and to stimulate B-lymphocyte proliferation.[125] It has also been shown to induce the secretion of platelet activating factor (PAF) by EC,[126] LTB_4 by macrophages,[118] a variety of cytokines by epithelial cells,[114-116] and to shed TNF receptors from phagocytes.[127] It has recently been reported that HNE can degrade the EC adhesion receptor, ICAM-1 and it has been suggested that in this way HNE might downregulate ICAM-1 mediated adhesion to neutrophils to EC.[128] However, another recent study reports upregulation of ICAM-1 expression by HNE (as determined both immunologically and by mRNA levels).[129] Taken together these studies raise the possibility of a negative feedback mechanism activated by the proteolysis of ICAM-1.

Several pro-inflammatory mediators as well as plasma proteins may be proteolytically modified by HNE, often with important physiological consequences. In this regard, HNE has been shown to generate IL-2 peptides which inhibit adherence of T-lymphocytes to the extracellular matrix.[130] HNE has also been shown to cleave IL-8 into a more active form,[131] to inactivate both TNFα and β,[132,133] and to generate complement components.[134,135] Proteolysis of clotting proteins by HNE is discussed in the following chapter (pages 81-84).

Until recently the biological relevance of HNE mediated proteolysis has been questioned, due to the relatively small quantities of HNE that are released from the neutrophil in response to biologically relevant stimuli,[2,136] and the high concentration of HNE inhibitors in the extracellular space.[137] (Whilst the most important of these

inhibitors is α_1-antiproteinase, formerly α_1-antitrypsin, HNE is also susceptible to other inhibitors including α_2-macroglobulin in the plasma and antileukocyte protease (ALP) in the mucosa).[137,138] Although the extracellular space is replete with high affinity HNE inhibitors, HNE in the immediate pericellular environment has been shown to retain its catalytic activity.[139-142] Numerous studies have investigated the factors responsible for this phenomenon, and a number of potential mechanisms have been identified:

It has been shown that reactive oxygen species produced by the MPO/H_2O_2/halide system are capable of oxidatively inactivating α_1-proteinase inhibitor.[143] Neutrophil and macrophage metallo-proteinases have also been shown to inactivate α_1-proteinase inhibitor.[144,145] Thus, proteolysis in the microenvironment surrounding the cell may be facilitated by local inactivation of proteinase inhibitors.

Another potential mechanism whereby HNE may be allowed to evade extracelllular inhibitors is through the creation of a microenvironment which excludes proteinase inhibitors. Neutrophils have been shown to form protected extracellular compartments between their plasma membranes and protein (e.g. fibrinogen, fibronectin and elastin) coated surfaces; these protected compartments can exclude most of the macromolecular components of plasma, including most naturally occurring proteinase inhibitors, thereby allowing continued proteolysis of the extracellular matrix.[139,141,146-148]

More recently HNE has been shown to evade inhibition by binding to the neutrophil surface.[51-53] Surface bound HNE retains its catalytic activity and is substantially resistant to inhibition by naturally occurring protease inhibitors.[51-53] Whilst resting neutrophils have been shown to express a significant level of HNE on their membranes,[52,54,113] this is greatly increased following neutrophil activation with the phorbol ester PMA, or the chemoattractants, fMLP or IL-8.[51-54] Priming of neutrophils with TNFα or PAF, prior to stimulation with these chemoattractants markedly enhances this response.[53] The fact that HNE expression is under the control of biologically relevant effector molecules suggests that the inducible

expression of membrane bound HNE may play an important role in the neutrophil inflammatory response.[53]

A comparison of protease-inhibitor and protease-substrate interactions, may help to explain the preservation of catalytic activity of surface bound proteases in the presence of naturally occurring protease inhibitors.[51] The complex formed between a proteinase and its inhibitor requires a very precise conformation and only one bond in the inhibitor molecule is subjected to cleavage. This contrasts with substrate hydrolysis where multiple bonds in the substrate molecule are accessible for cleavage. Thus steric hindrances would have a far greater effect on inhibitors attempting to interact with the active site of membrane bound proteases than substrates.[51]

There are numerous examples of membrane (or biological surface) associated proteinases that remain functional whilst demonstrating relative resistance to naturally occurring inhibitors. One such example includes urokinase plasminogen activator (u-PA), which converts the zymogen plasminogen to the active enzyme plasmin. Interestingly, plasmin also displays substantial resistance to its natural inhibitor, (α_2-antiplasmin) when membrane bound.[149,150] Several instances where proteases (including HNE) become resistant to their natural inhibitors when bound to *fibrin*, are described in the following chapter.

In addition to conferring resistance to inhibition, the binding of HNE to the neutrophil membrane also helps to focus its activity in the immediate pericellular environment and to prevent its diffusion from the vicinity of the neutrophil. This allows HNE mediated proteolysis to be both highly localised and tightly regulated.[53] The neutrophil membrane has been reported to contain receptors that are specific for HNE,[151] and more recently, HNE has been shown to bind specifically to Mac-1 coated surfaces.[113] It has, however, been suggested that the association of this protease with the neutrophil membrane is largely non-specific, and due to the interaction between negative charges on the neutrophil surface (sialic acid residues) and positive charges on HNE.[51] This is supported by the recent observations that cationic proteins abrogate the agonist induced upregulation of HNE

expression on the neutrophil surface, and that HNE can be eluted off the neutrophil surface by solutions of increasing ionic strength.[53]

Recent studies therefore suggest that *membrane-bound* HNE is an important biological form of this protease, allowing its catalytic activity to be preserved in the presence of naturally occurring proteinase inhibitors, and to be localised to the immediate pericellular environment.

2. Cathepsin G

Cathepsin G is a 29 kDa glycoprotein which, like HNE, is a cationic serine protease of relatively broad substrate specificity. It is often referred to as a chymotrypsin-like enzyme because it hydrolyses peptide bonds after leucine, methionine and phenylalanine residues.[152] Relative to HNE, cathepsin G is considered to be a rather inefficient proteinase, degrading a number of substrates more slowly than HNE.[152,153] Whilst its natural inhibitor is α_1-antichymotrypsin,[51] cathepsin G is also very susceptible to α_1-antiproteinase.[154] Like HNE, cathepsin G is also able to evade the action of naturally occurring proteinase inhibitors through its ability to bind to the neutrophil membrane,[51,52] and binding appears to be mediated via a similar charge related mechanism.[51]

Cathepsin G is a multifunctional protease which shares a number of functions with HNE. These include the upregulation of the neutrophil respiratory burst,[119] the stimulation of B-cell proliferation,[125] the modification of cytokines such as IL-8[131] and the degradation of a variety of extracellular matrix proteins including fibronectin, fibrinogen, immunoglobulins and various complement components.[155-158] In addition, cathepsin G possesses microbicidal activity which is more potent than that of HNE.[19]

Cathepsin G also has a number of unique actions, including chemotactic activity for monocytes and neutrophils,[159] the activation of HNE,[160] and the induction of platelet activation and aggregation.[161-163] The latter effect is substantially enhanced in the presence of HNE.[124,163]

Recent studies have shown that certain synthetic peptides derived from C-reactive protein (CRP) can act as inhibitors of both HNE and cathepsin G.[164,165] As CRP is a known substrate for a recently identified neutrophil membrane associated protease (see page 34), it is possible that neutrophil mediated proteolysis of CRP, through the generation of inhibitory peptides, might regulate the proteolytic activity of HNE and cathepsin G.

3. Proteinase 3

Proteinase 3 (PR3) was the third serine protease to be identified in azurophil granule.[166] This 29 kDa glycoprotein is a major constituent of the azurophil granule, being present in amounts comparable to that of HNE.[43,167] PR3 shows a preference for peptide bonds involving small aliphatic amino acids such as alanine, serine and valine.[168]

PR3 has extensive NH_2-terminal homology with HNE and cathepsin G, and shows extensive *active site* homology with HNE. [168,169] It is thus not surprising that these proteases are very similar in function. Like HNE and cathepsin G, PR3 is capable of degrading elastin as well as a wide variety of connective tissue proteins, including fibronectin, laminin, vitronectin, and collagen type IV.[168] This suggests that PR3 also has the potential to cause extensive tissue destruction. Indeed, this has been demonstrated in animal models, where the capacity of PR3 to cause emphysema was shown to be similar to that of HNE.[165]

PR3 may also perform a number of important biological functions, including the modulation of cathepsin G induced platelet activation[171] and the proteolytic modification of IL-8 into more active forms.[131] In addition, it has potent microbicidal activity against bacteria and fungi.[43,170] Whilst PR3 is inhibited by α_1-antiproteinase as well as α_2-macroglobulin, it is not sensitive to either α_1-antichymotrypsin or antileukoproteinase.[168]

The link between PR3 and Wegener's granulomatosis (a severe, destructive disease characterised by necrotising vasculitis and granuloma formation) has generated substantial interest in this

protease. Wegener's granulomatosis is associated with the presence of circulating antibodies against PR3.[173,174] Whilst the detection of antibodies in the serum of patients assists in the diagnosis of this disease, the precise role of these antibodies in the pathogenesis of Wegener's granulomatosis is not entirely clear.[174]

Like HNE and cathepsin G, PR3 has also been shown to bind to the neutrophil membrane following release by activated neutrophils.[172]

4. Azurocidin

Although a member of the family of hematopoeitic serine proteases, azurocidin is a "sterile" enzyme which lacks proteolytic activity due to mutations in 2 of 3 residues in the presumed catalytic triad of the protein; the histidine (His_{41}) is replaced by a serine residue and the serine (Ser_{175}) is replaced by a glycine residue, whilst the aspartate (Asp_{89}) is conserved. [92,93] The functional gene for azurocidin is grouped in a cluster of elastase like genes within a 50 kb region on chromosome 19.[175]

This 29 kDa glycoprotein is relatively abundant in azurophil granules,[176] and despite its lack of catalytic activity, azurocidin has been shown to have other important properties. It is a potent antibiotic against Gram negative bacteria and a major contributor to the overall antimicrobial activity of the neutrophil azurophil granule *in vitro*.[43,170] Azurocidin also appears to function as a strong chemotactic agent for monocytes and neutrophils.[159,177] Its poor solubility at neutral pH under non-denaturing conditions has led to the suggestion that may be associated with the granule membrane.[176]

II. Neutrophil matrix metalloproteinases

Matrix metalloproteinases (MMP) are a highly homologous family of zinc and calcium dependent endopeptidases, which play an important role in the degradation of extracellular matrix proteins.[178,179] The MMP gene family includes gelatinases (MMP-2 and MMP-9), collagenases (MMP-1, MMP-8), stromelysins (MMP-3, MMP-10, MMP-11), PUMP-1 (MMP-7) and membrane type MMP (MT-

MMP).[180-183] The neutrophil contains two MMPs, namely neutrophil gelatinase (MMP-9) and neutrophil collagenase (MMP-8). These proteases are secreted as zymogens which may be activated by oxygen metabolites or proteolytic processing,[18] and under certain conditions, by autocatalytic cleavage.[181,182]

1. Neutrophil gelatinase

Neutrophil gelatinase (MMP-9) is a 92 kDa protein found in the specific (secondary) and gelatinase (tertiary) neutrophil granules.[2] Whereas gelatinase in the specific granules is present in relatively low concentrations and partially complexed to a 25 kDa protein NGAL (neutrophil gelatinase associated lipocalin), gelatinase in the tertiary granules is stored in high concentrations in its free form.[2]
Gelatinase is initially mobilised from the tertiary granules by chemotactic signals during neutrophil recruitment,[31,67] and is secreted as a latent pro-enzyme, which is activated by oxygen metabolites or proteases, including HNE and cathepsin G.[18,54,184]
Neutrophil gelatinase is able to degrade the major components of the endothelial basement membranes and extracellular matrix, namely native or denatured type IV collagen/gelatin, laminin, and fibronectin.[185] Numerous reports suggest that gelatinase plays an important role in neutrophil migration.[72,73,186] It has recently been shown that, following release, gelatinase can bind to the neutrophil membrane where it becomes activated by membrane bound HNE and cathepsin G.[54] This may have important implications for the regulation of neutrophil migration through connective tissue.

2. Neutrophil collagenase

Collagenase is a 75 kDa protease which preferentially degrades native type I collagen.[180,185] Neutrophil procollagenase is stored as a glycosylated protein within the specific granules of the neutrophil.[187,188] Inflammatory mediators induce the release of procollagenase[189] which can then be activated by the neutrophil, in a similar manner to that described for gelatinase. The activated protease plays a key role in connective tissue turnover due to its ability to degrade collagen in the extracellular matrix.

Serine proteases of the neutrophil membrane

Over the last decade, there have been numerous reports of serine protease activity associated with the neutrophil membrane. This review considers three neutral serine proteases which have been reported to date.

1. 300 kDa protease

Pontremoli et al[190] reported a novel 300 kDa membrane which was distinguished from serine proteases of the neutrophil azurophil granule on the basis of its molecular weight, inhibitor profiles and substrate specificities.[190] Stimulation with low dose PMA (10ng/ml) induced release of this protease into the extracellular medium. This concentration of PMA was reported to cause only a minor discharge of secondary granules and no release of azurophil granules (as indicated by the absence of azurophil granule markers β-glucuronidase and N-acetylglucosaminidase). The release the 300 kDa protease from the neutrophil was ATP dependent and associated with phosphorylation of membrane proteins.[191] This protease, located at sites of interaction of the neutrophil membrane and cytoskeleton,[192] was reported to have cytolytic activity[190] and to downregulate protein kinase C activity.[192]

2. 65-70 kDa protease

King et al[193] identified a membrane associated serine protease which was immunoprecipitated by an antibody which recognises chymotrypsin. Although this "chymotrypsin-like" protease was originally reported to have a molecular weight of 150-180 kDa, immunoaffinity chromatography later revealed it to be 65-70 kDa in size.[194] It was suggested that this discrepancy in size was due to the propensity for these molecules to form dimers or trimers under the original experimental conditions.[194] The authors pointed to previous studies which noted a tendency for membrane proteins to aggregate after solubilisation unless maintained in conditions of high osmolarity.[195,196] Interestingly, the proteolytic activity of this protease

was noted to be very similar to that of cathepsin G with respect to its inhibitor profile, its optimal pH, its affinity for substrates containing aromatic amino acids, and its requirement for calcium as a co-factor. However, it was suggested that it was unlikely that the detected proteinase activity was due to contamination with cathepsin G as the starting membrane preparation was free of other azurophil granule markers.[196] The recent observations, however, that the highly cationic azurophil granule serine proteases can bind to the neutrophil membrane by a charge related mechanism, brings into question the validity of using markers such as myeloperoxidase (MPO) or β-glucuronidase to exclude the involvement of granule enzymes. During preparative procedures which involve cell lysis, the liberated cationic serine proteases may have a high propensity for binding to the membrane fraction, which is not neccessarily shared by the commonly used markers such as MPO and β-glucuronidase. Thus, it is possible that membrane preparations shown to be free of azurophil granule markers, may in fact be contaminated with azurophil granule serine proteases.

3. 600 kDa protease

A 600 kDa neutral serine protease of the neutrophil membrane has recently been reported to degrade C-reactive protein (CRP).[197] This protease was found to be associated with the neutrophil membrane and cytoskeleton fractions, and was partially released in response to stimulation with a dose of PMA (10ng/ml) which has been reported not to induce the release of azurophil granules. [190,197] The proteolytic activity of the neutrophil membrane and cytoskeleton fractions, as well the conditioned medium from PMA stimulated neutrophils, was shown by size exclusion chromatography to elute in a single peak corresponding to a molecular weight of 600 kDa. When each of these three fractions were subjected to SDS/PAGE, the proteolytic activity in each fraction, responsible for degrading CRP, was reported to migrate as four discrete bands to positions corresponding to apparent molecular mass values of 200 kDa, 316 kDa, 398 kDa and 501 kDa. It was suggested that these represented proteolytically active subunits of the 600 kDa protein.[197]

Each fraction (membrane, cytoskeleton and PMA stimulated conditioned medium) was reported to generate CRP degradation products with identical apparent molecular mass values. These products were reported to differ in molecular mass from those produced by the action of azurophil granule (lysosomal) proteases on CRP. The CRP degrading activity of both the cytoskeleton and membrane fractions was reported to be completely inhibited by PMSF implying that this enzyme was a serine protease.[197]

These same neutrophil fractions were subsequently reported to possess fibrinogen and fibrin degrading activity.[198,199] Once again, when subjected to SDS/PAGE, the proteolytic activity in each fraction, responsible for degrading fibrin(ogen), was reported to migrate as four discrete bands to positions corresponding to apparent molecular mass values of 200 kDa, 316 kDa, 398 kDa and 501 kDa.[198,199] This suggested that the same protease degraded CRP, fibrinogen and fibrin. This protease was reported to generate fibrin(ogen) degradation products with distinctly different molecular mass values from those produced by plasmin, neutrophil lysosomal enzymes and purified HNE.[198,199] Inhibitor profiles also suggested that this protease was distinct from known neutrophil proteases. Two specific chloromethyl ketone inhibitors of HNE (namely Suc-$(Ala)_3$-CH_2Cl and Suc-$(Ala)_2$-Val-CH_2Cl) were reported to have no effect on fibrinogenolysis by these neutrophil preparations, whilst a third, MeO-Suc-$(Ala)_2$-Pro-Val- CH_2Cl, was reported to be only partially inhibitory (50% at 20 hours). Likewise, a specific inhibitor of cathepsin G, Suc-$(Ala)_2$Pro-PheCH_2Cl, was reported to be non-inhibitory.[198] As with CRP, fibrin(ogen) degrading activity was very sensitive to inhibition by PMSF (phenylmethylsulphonyl fluoride) and α_1-antitrypsin, but resistant to a number of cysteine and metalloproteinase inhibitors. This protease was reported to cleave a peptide from the N-terminus of the Aα chain of fibrinogen (the Aα 1-21 peptide), previously thought to be produced exclusively by HNE.[198]

Neutrophil adhesion

The ability of cells to adhere to one another and to proteins in the extracellular matrix is central to a variety of important biological processes, including cell differentiation, migration, wound healing, tumour invasion, and cellular responses to inflammation.[200] In the neutrophil, adhesive interactions are regulated by three distinctive families of adhesion receptors, namely the integrins, the selectins and the immunoglobulin superfamily. Each class of receptor will be reviewed in turn after which their collective roles in the adhesion process will be considered with particular reference to *neutrophil-endothelial cell* interactions and *neutrophil-extracellular matrix* interactions.

1. Integrin receptors

Integrins are a ubiquitous family of highly differentiated adhesion molecules which direct the processes of cell-cell communication as well as cellular interaction with the extracellular matrix.[201] These membrane glycoproteins also serve as transmembrane links between the extracellular environment and the cytoskeleton of the cell,[202,203] and are thus able to act as signal transducers modulating important cellular functions.[200,204] As such they are intimately involved in many complex processes beyond adhesion including haemostasis, cell maturation and embryogenesis.[200,205]

In addition, integrins are able to modulate a variety of important leukocyte inflammatory responses such as cytokine production,[206] phagocytosis and the respiratory burst.[207,208] Integrins are heterodimeric transmembrane proteins composed of non-covalently linked α- and β-subunits (Figure 12). These subunits are the products of two different genes,

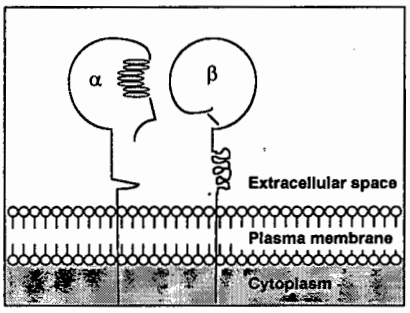


***Figure 12**: A schematic representation of the two subunits of the heterodimeric integrin receptors.*

and the expression of both subunits is required for receptor function.[209] To date the integrin superfamily is known to contain 16 different α-subunits and 8 different β-subunits. These can pair in various combinations to form one of 22 different of αβ-dimer permutations.[200] Integrins are divided into subclasses according to their β-subunit, and at least 8 subclasses (β_1-β_8 intergrins) are known to exist.

Neutrophils express three subclasses of integrin receptors, namely β_1, β_2, and β_3-integrins. The β_2-integrin subclass is unique to leukocytes and the expression of this subset is far higher than the other two subclasses.[210] These receptors, also known as the *leukocyte integrin family*, will be the major focus of the review which follows. Beta-1-integrins, also known as very late antigens (VLAs), are the most widely distributed class of integrin receptors in mammalian cells;[211] in neutrophils $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_4\beta_1$, $\alpha_5\beta_1$ and $\alpha_6\beta_1$ are expressed.[210] The β_3-integrin family of receptors, also known as cytoadhesins, include the leukocyte response integrin (LRI), the vitronectin receptor as well as the platelet receptor, GPIIb/IIIa.[205,212]

Electron microscopy has revealed integrins to be asymmetric structures consisting of a mushroom-like extracellular region (formed by the NH_2-terminal regions of the α and β subunits) and two flexible cytoplasmic tails (formed by the C-termini of these subunits).[213] The extracellular domains of the α and β subunits associate with each other to form the ligand binding pocket,[211] whilst the cytoplasmic domains are thought to be linked to the cytoskeleton through intermediary linker proteins such as talin and α actinin.[214] This arrangement is believed to facilitate the translation of the extracellular signal, induced by ligand binding, to an intracellular event via reorganisation of the cytoskeleton. Integrins can bind to a variety of ligands including extracellular matrix (ECM) proteins,[215] cell surface immunoglobulin superfamily receptors,[216] and certain plasma proteins.[217] ECM ligands for integrins include fibronectin, fibrinogen, laminin, collagens, entactin, tenascin, thrombospondin and vitronectin.[202,218,219] Certain integrin receptors can bind more than one ECM, whilst certain ECM proteins can bind more than one integrin receptor. This results in considerable overlap and redundancy in integrin-ligand interactions.

Divalent cations regulate the binding of integrins to the majority of their ligands,[220,221] and integrin mediated cell adhesion, as well as ligand binding to purified receptor, is dependent on millimolar concentrations of calcium.[222-224] Inactivated integrins usually have a low affinity for ligand; however, upon activation via appropriate signals, integrins can participate in high affinity binding.[200,220] This unique function allows for the modulation of cell adhesive properties without quantitative upregulation of integrin expression on the cell surface.[11,200,225] Integrin receptors frequently recognise their ligands via the tripeptide amino acid sequence Arg-Gly-Asp (RGD),[202] although β_2-integrins are able to recognise a variety of other peptide sequences as well.

Leukocyte integrins

The β_2-integrins are by far the most abundant and important subset of integrin receptors on the neutrophil,[210] and are thus the major focus of the review which follows.

Beta-2-integrins, frequently referred to as leukocyte integrins, comprise a group of closely related heterodimeric molecules which can regulate the leukocyte inflammatory response.[220] These glycoproteins share a common β (CD18) subunit, and are distinguished by their α (CD11)-subunits, namely CD11a, CD11b, CD11c, CD11d.[226,227] The four heterodimers are thus referred to as CD11a/CD18 (or lymphocyte function associated antigen [LFA]-1), CD11b/CD18 (or Mac-1), CD11c/CD18 (or p150,95) and CD11d/CD18 ($\alpha_D\beta_2$). The important role played by the leukocyte integrins in the inflammatory response is highlighted by the severe and recurrent infections suffered by patients who lack these receptors.* The expression of each β_2-integrin subset varies with the the different leukocyte subpopulations. CD11a/CD18, whilst present to some extent on all leukocytes, is primarily expressed on lymphoid cells. On the other hand CD11b/CD18 is enriched on cells of the myeloid lineage whilst CD11c/CD18 and CD11d/CD18 are most

* Leukocyte adhesion deficiency (LAD) is a syndrome associated with a deficiency of leukocyte β_2-integrins due to a mutation in the gene encoding CD18.[226,228]

strongly expressed on monocytes and macrophages.[220] All subclasses are expressed, to varying degrees, on neutrophils.

The polypeptide chains comprising the α and β-subunits are type 1 membrane proteins. The α-subunits comprise a short cytoplasmic tail, a transmembrane domain and a large extracellular domain of approximately 1000 amino acids. (see figure 13). This extracellular domain contains 7 tandem repeats of 60 amino acids which contain divalent cation binding site. [200] Within these repeats is an intervening I-domain, of approximately 200 amino acids, which contains the ligand binding sites critical for β_2-integrin receptor function.[229] The cytoplasmic tail of the α-subunit contains a conserved basic sequence near the transmembrane region, GFFKR, which is thought to play an important role in maintaining neutrophils in an inactive state in the absence of stimuli.[230] The extracellular domain of the β-subunit contains a region of approximately 200 amino acids which shows similarities to the I domain of the α-subunit, and just proximal to the membrane, a region comprising four cysteine rich motifs. Like the α-subunit, the β-subunit also has a short cytoplasmic tail. [200,210,220]

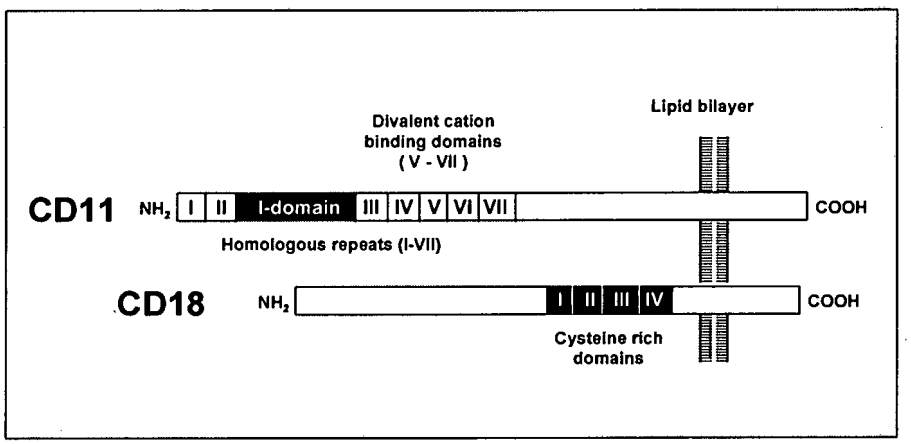


Figure 13: *A schematic representation of the structure of the β_2-integrins. The CD11 (α-chain) contains the ligand binding I-domain as well as divalent cation binding domains. The CD18 (β-chain) is non-covalently associated with the α-chain to form the functional β_2-integrin receptor.*

The adhesiveness of leukocyte integrins is not constitutive but dependent upon receptor activation.[225,231] Recent evidence suggests

that β_2-integrins may be activated either directly, via ligand binding to integrin receptors, or indirectly, as a consequence of ligand binding to non-integrin molecules.[220] Whilst the mechanisms behind direct activation are still relatively poorly understood, indirect activation has been far more extensively studied and several known mediators exist. These include various inflammatory mediators such as TNF, IL-8, C5a, PAF and FMLP as well as non-physiological activators, such as the phorbol ester, phorbol-3-myristate-4-acetate (PMA).[11,225] It has been suggested that the binding of these mediators sets in motion a cascade of signalling events which ultimately leads to phosphorylation of serine/threonine residues in cytoplasmic tail of CD18, thereby activating the receptor.[220] Despite intensive research in this area, a more complete understanding of the mechanisms behind of integrin activation remains to be achieved.[220] The four β_2-integrin subsets are reviewed in turn below.

1. CD11a/CD18 (LFA-1)

This integrin receptor, also known as lymphocyte function associated antigen-1 (LFA-1) is expressed on all leukocytes, but is primarily associated with lymphocytes.[226] CD11a/CD18 mediates leukocyte adhesion to endothelial cells,[11] and supports T lymphocyte helper and killer function.[232] Ligation of CD11a/CD18 by ICAM-1 has recently been reported to signal for neutrophil phagocytosis.[233]

2. CD11b/CD18 (Mac-1)

CD11b/CD18, often referred to as Mac-1 or CR3, is enriched on granulocytes. It is arguably the most important and versatile adhesion receptor on the neutrophil, and is intimately involved in a number of fundamental cellular processes. Mac-1 plays an important role in neutrophil adhesion to, and transmigration across the endothelium.[234,235] It also mediates neutrophil homotypic aggregation,[236,237] chemotaxis,[236] phagocytosis,[238-240] and adherence to serum coated substrates.[236] These Mac-1 dependent adhesive interactions result from its ability to bind to a variety of cellular and soluble ligands including C3bi,[241,242] intercellular adhesion molecule 1 (ICAM-1),[225,234] fibrinogen,[243,244] factor X,[245] and L-selectin.[246]

The I domain of Mac-1 has been shown to be critical for the recognition of most of its ligands.[229]

Mac-1 does not bind to its ligands until a specific stimulus has been delivered. The increased avidity which follows Mac-1 activation has recently been attributed to the expression of an activation specific neoepitope on a small subset of Mac-1 receptors (10-30%).[225] Monoclonal antibodies directed against this epitope have been shown to inhibit neutrophil adhesion to ICAM-1 and fibrinogen by over 90%.[225]

3. CD11c/CD18 (p150,95)

The expression of CD11c/CD18 is mainly restricted to neutrophils and monocyte/macrophages, although it has been identified on some lymphocytes, and is a marker of hairy cell leukaemia.[247-249] In contrast to CD11a/CD18 and CD11b/CD18, the functional role of CD11c/CD18 is not clearly understood. Like the other leukocyte integrins, this receptor is involved in neutrophil adherence to endothelium,[249,250] binds to fibrinogen[251] and C3bi opsonised particles,[252] and more recently has been identified as a transmembrane signalling receptor for lipopolysaccharide (LPS).[253]

4. CD11d/CD18

The most recently identified member of the β_2-integrin family is CD11d/CD18.[227] The CD11d subunit resembles CD11b and CD11c more closely than CD11a. It differs in its distribution from the other leukocyte integrins in that it is expressed on granulocytes and macrophages in the splenic red pulp, on lipid laden macrophages in aortic fatty streaks and to a lesser extent on peripheral blood leukocytes.[221] The only known ligand for this receptor identified to date appears to be ICAM-3 which is constitutively expressed on resting leukocytes.[221]

Leukocyte response integrin (LRI)

Leukocyte response integrin (LRI), which shows strong homology to members of the β_3-integrin subfamily, is one of the more recently characterised neutrophil integrin receptors.[205,254] Unlike members of

the β_2-integrin subfamily, this receptor does not require prior activation in order to bind to its ligands. It is intimately associated with a 50 kDa protein, known as integrin associated protein (IAP) and together they form a signal transduction unit for the activation of phagocytes.[255,256] Several proteins containing RGD-sequences have been reported to transduce signals for neutrophil activation via LRI and IAP. These include entactin, fibrinogen, fibronectin, von Willebrand's factor, vitronectin and collagen type IV.[205] As the binding of the RGD-sequence to LRI has been shown to serve as a signal for both chemotaxis[257] and phagocytosis,[205] it has been suggested that this receptor may play an important role in the neutrophil inflammatory response.

2. Selectins

The selectins are a family of three integral membrane glycoproteins. Each member of this family has an amino terminal lectin domain, an epidermal growth factor (EGF)-like sequence, a transmembrane domain, and a short cytoplasmic tail (see figure 14).[258,259] The lectin and EGF-like domains are critically involved in adhesion.[259,260] These domains bind to sialylated glycoproteins on the surface of target cells.[259] L-selectin is found on the surface of leukocytes and E-selectin on endothelial cells (EC), whilst P-selectin was initially identified on platelets and later on EC.[259] Tethering interactions between selectins and their ligands (sialylated glycoproteins) on the neutrophils and EC) cause neutrophils to slow down and roll along the vessel wall.[261,262] Selectins are ideally suited to this tethering role owing to their long molecular structure that extends above the surrounding glycocalyx

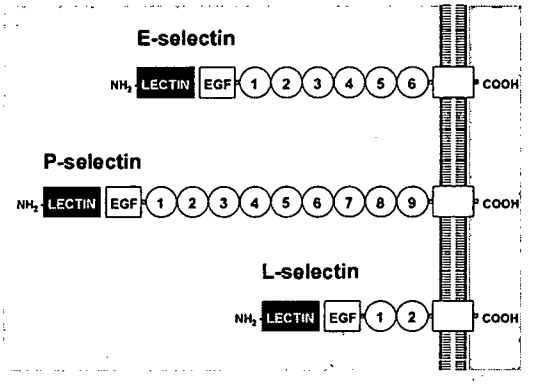


Figure 14: *A schematic representation of selectin structure indicating the amino terminal lectin domain, EGF repeat, the complement regulatory-like molecules the transmembrane domain and the cytoplasmic tail.*

and allows them to capture passing leukocytes that express the appropriate receptor.[262] Selectin mediated adhesion is strong enough to induce rolling along the vessel wall but not so strong as to stop leukocytes completely.[262] This does however allow neutrophils to be exposed to potential integrin activating agents such as chemokines, formylated bacterial peptides (e.g. FMLP) and complement fragments.[210] Recent studies suggest that, in addition to their adhesive functions, selectins may directly signal cell responses. Neutrophils adherent to E-selectin on IL-1 prestimulated EC, or to preparations of recombinant E-selectin adsorbed to a plastic surface, are reported to increase the adhesiveness of Mac-1.[263,264] In addition it has recently been reported that crosslinking of L-selectin signals for the upregulation of Mac-1 dependent adhesion.[265]

The various selectins differ from one another in their pattern of expression. L-selectin is constitutively expressed on most leukocytes, and upregulation occurs through the rapid enhancement of the avidity of L-selectin for its counter receptors.[266] L-selectin is rapidly shed from the plasma membrane during the process of neutrophil activation following proteolytic cleavage near its transmembrane region.[266,267] E-selectin is not constitutively expressed by the EC but is actively synthesised following EC activation with various chemokines.[268] P-selectin, which is stored in EC secretory granules, is rapidly translocated to the plasma membrane following EC stimulation with thrombin or histamine.[262] Thereafter it is rapidly re-internalised, resulting in its transient expression which parallels neutrophil adhesion to activated EC. The transience of P-selectin expression provides a mechanism to temporally regulate such adhesive interactions.[269,270]

3. Intercellular adhesion molecules (ICAM)

Intercellular adhesion molecules (ICAM) are the EC counter-receptors for activated leukocyte β_2-integrins, and as such play an integral role in neutrophil-EC interactions. ICAMs belong to a broader family of adhesion receptors, known as the immunoglobulin superfamily, so named due to the repeated immunoglobulin-like regions in their extracellular domains (Figure 15). There are three

members of the ICAM family, namely ICAM-1, -2 and -3. ICAM-1 and 2 are involved in neutrophil-EC interactions, and these two transmembrane glycoproteins are closely related in terms of structure and function.[271] Whilst ICAM-1 contains 5 extracellular immunoglobulin (Ig)-like domains, ICAM-2 contains only two. The two Ig domains of ICAM-2 show strong homology with the two amino-terminal domains of ICAM-1 (which are known to mediate ligand binding), and this may explain their partially overlapping functions.[272,273] ICAM-2 is constitutively expressed on EC, and therefore mediates binding to resting endothelium. In contrast, ICAM-1 expression, which is very low in resting cells, is markedly upregulated following EC activation and is therefore involved in neutrophil binding to inflamed endothelium.[259,271] ICAM-1 is recognised by both CD11a/CD18 and CD11b/CD18, whereas ICAM-2 is recognised only by CD11a/CD18.[271,274] Ligation of CD11a/CD18 and CD11b/CD18 by ICAM-1 has recently been shown to upregulate the release of HNE by the neutrophil with an associated increase in neutrophil mediated cytotoxicity.[275]

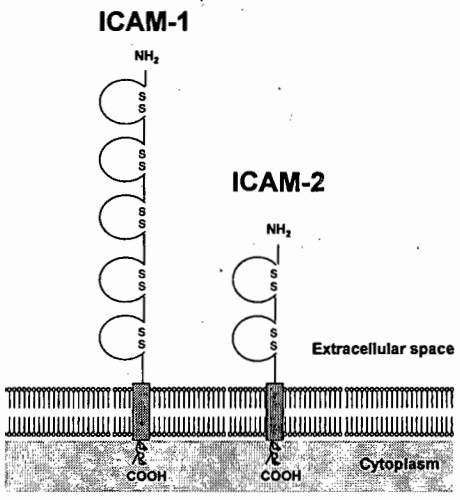


Figure 15: *A schematic representation of ICAM 1 and 2, showing the extracellular immunoglobulin like domains.*

Neutrophil-endothelial cell interaction

An early and requisite step in the acute inflammatory response is the migration of neutrophils from the circulation into sites of inflammation. This requires that the circulating neutrophil makes contact with the endothelium, slows down, becomes adherent and finally migrates through the blood vessel.

The adhesion cascade is initiated by tethering interactions between neutrophils and EC which cause circulating neutrophils to slow down. This is mediated by selectins both on the neutrophil and on EC, which interact with sialylated carbohydrate residues on target cells. This results in a degree of adhesion that is sufficient to induce neutrophil rolling on the vessel wall but not strong enough to halt them completely.[261,262] This transient interaction allows neutrophils to become exposed to various factors on the EC surface that activate neutrophil expression of β_2-integrins. Two such factors include IL-8 and PAF, which may be produced by activated EC or underlying inflammatory cells.[11,276,277] The immobilisation of these proadhesive cytokines on the EC surface may localise leukocyte activation to areas of endothelium overlying inflammatory sites, and prevent the removal of cytokines in passing blood. Other potential trigger factors for β_2-integrin activation include bacterial wall components (eg. FMLP and LPS), complement products,[211] and selectins themselves.[263-265] These factors signal for both *quantitative* upregulation of β_2-integrin expression as well as a *qualitative* alteration of the receptors constitutively present in the plasma membrane.[11] The latter appears to be the primary mechanism whereby neutrophil adherence to the endothelium is increased, as suggested by two series of observations. Firstly, maximal adherence of activated neutrophils to EC has been shown to precede the maximal recruitment CD11b/CD18 to the surface, and secondly, the adhesive interaction reverses during a period when increased numbers of this heterodimer are present on the neutrophil surface.[11] This functional upregulation has recently been attributed to an activation specific epitope on CD11b/CD18 (see page 41).[225] Similarly, CD11a/CD18 has also been shown to undergo functional upregulation, which is mediated via alterations of the cytoplasmic domain of its β-chain.[278,279] This is important since this receptor does not appear to undergo quantitative upregulation following neutrophil activation.[280] The binding of β_2-integrins to their counter-receptors, ICAM-1 and ICAM-2, on EC mediates strong adhesion between neutrophils and the endothelium.[11,271]

Strong adhesion is a transient phenomenon, and following weakening of integrin mediated adhesion, neutrophils migrate into the interstitium.[220] Of all the steps involved in neutrophil migration to extravascular sites of inflammation, transendothelial migration is the

least understood.[211] It has been suggested that reciprocal interactions between CD31 and $\alpha_v\beta_3$ (which are present on both neutrophils and EC) may facilitate this process, although the precise mechanisms involved remain unclear.[211] Recent studies suggest that neutrophil elastase (HNE) may play an important role in transmigration, not only through its ability to digest the basement membrane, but also through its ability to release integrins from their substrates.[112,116]

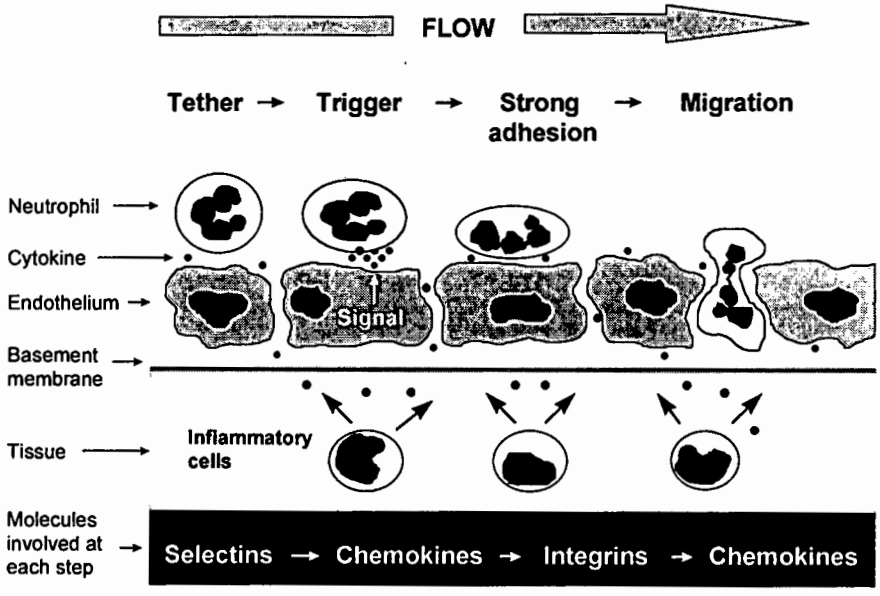


Figure 16: *The sequential steps in the adhesion of neutrophils to activated endothelium. The circulating neutrophil is tethered and brought into contact with the EC wall by selectin mediated interactions. Tethering allows for the exposure of neutrophils to cytokines released by underlying inflammatory cells and expressed on the EC surface. Cytokines trigger the expression and upregulation of integrin receptors which facilitate strong adhesion of neutrophils to EC. The transience of this interaction allows for the subsequent migration of neutrophils into the underlying tissue under the influence of chemoattractants released by underlying inflammatory cells or invading micro-organisms.*

Neutrophil-extracellular matrix interactions

Having traversed the endothelium, the neutrophil must next migrate through the extracellular matrix (ECM) to the site of tissue damage. The ECM is a relatively stable structural matrix which underlies epithelia and endothelia and surrounds connective tissue cells. It is composed of collagens, glycoproteins, proteoglycans, elastin and associated molecules such as fibronectin, vitronectin, fibrinogen, and laminin. Once regarded solely as an extracellular scaffold for the maintenance of tissue architecture, the ECM is now recognised as a highly interactive substratum capable of modulating cellular function. The interaction between neutrophils and the ECM may have a profound influence of the neutrophil inflammatory response.

Whilst fibrinogen is the major ECM protein recognised by β_2-integrins,[211] the β_1 and β_3-integrins recognise a wide variety of ligands containing the tripeptide sequence Arg-Gly-Asp (R-G-D), including fibrinogen, fibronectin, thrombospondin, vitronectin, entactin and collagen type I and IV.[211,254] Integrin receptors provide the link between the extracellular environment and the cytoskeleton, with ligation of these receptors signalling for important cellular functions.

When neutrophils adhere, via β_2-integrin receptors, to ECM protein coated surfaces, they undergo a number of functional changes - adherent neutrophils become far more reactive to inflammatory stimuli than those in suspension.[207,208] The influence of β_2-integrin mediated adherence on neutrophil function was first noted with respect to the respiratory burst. Whilst neutrophils in suspension were shown to be poorly responsive to stimulation with low dose TNFα and β, those adherent to the ECM respond with a full scale respiratory burst.[207] The β_2-integrin dependence of this phenomenon was suggested by two sets of observations. Firstly, neutrophils from patients with leukocyte adhesion deficiency (LAD), whose leukocytes are deficient in CD18, were found not to undergo a normal cytokine induced respiratory burst on ECM coated surfaces. Secondly, neutrophils adherent to surface-bound *anti-CD18 antibody* were noted to respond with a respiratory burst similar to that seen in ECM adherent neutrophils, following cytokine stimulation.[208]

Neutrophil spreading on the ECM has subsequently been shown to be a prerequisite for the adhesion-dependent respiratory burst.[282-284] Both β_2-integrin mediated adhesion and respiratory burst have been shown to be linked to protein tyrosine phosphorylation - tyrosine kinase inhibitors are capable of blocking both these responses,[283,285] whilst β_2-integrin dependent adhesion to the ECM triggers tyrosine phosphorylation of a variety of neutrophil proteins.[285,286] As tyrosine phosphorylation is an important signalling mechanism in neutrophil activation,[287-289] the increased levels of tyrosine phosphorylation triggered by β_2-integrin mediated adherence is likely to influence a variety of important neutrophil responses. Indeed, in addition to its effect on the respiratory burst, β_2-integrin mediated adhesion upregulates several other neutrophil inflammatory responses. This includes the release of specific granules from the neutrophil,[290] which as noted earlier, alters the membrane phenotype cell and therefore a variety of inflammatory functions. Beta-2-integrin mediated adhesion also appears to upregulate neutrophil cytokine production, as suggested by the enhanced production of IL-8 noted in *adherent* neutrophils stimulated with LPS.[206] As IL-8 is a potent chemoattractant for neutrophils, this may have important consequences for neutrophil recruitment to sites of inflammation. Whilst the molecular events involved in signal transduction from the integrin receptor to the cytoskeleton will not be addressed here, a recent review suggests these processes are both highly complex and incompletely understood.[200]

Neutrophil interaction with fibrinogen

Many of the studies investigating the effect of β_2-integrin-dependent adhesion on neutrophil function (see above) have used fibrinogen as the ECM substrate.[206,208,286,290] This suggests that surface adherent fibrinogen has the ability to modulate neutrophil function at inflammatory sites.

On the neutrophil, the β_2-integrins, CD11b/CD18 and CD11c/CD18, as well as the β_3-integrin, LRI, serve as receptors for fibrinogen.[244,251,254] The nature of the stimulus determines which of the β_2-integrin receptors will bind fibrinogen.

- Neutrophils stimulated with the phorbol ester PMA bind to surface adherent fibrinogen via the CD11b/CD18 (Mac-1) integrin receptor.[244] This was initially reported to occur via the K-Q-A-G-D-V sequence situated at the carboxyl terminus of the fibrinogen γ-chain, as suggested by studies using peptide mimicry.[244] It was later shown that, in neutrophils stimulated with the chemotactic peptide FMLP, Mac-1 recognises fibrinogen via a different region of the γ-chain, as suggested by the inhibition of neutrophil binding to both soluble and surface adherent fibrinogen by peptides corresponding to the γ-chain sequence Gly_{190}-Val_{202}.[281] More recently, it has been suggested that this region together with the γ-chain region γ-377-395 forms a binding pocket for the I-domain on CD11b/CD18.[291]

- Neutrophils stimulated with TNFα, have been reported to bind to fibrinogen coated surfaces via CD11c/CD18, interacting with the G-P-R sequence at positions 17-19 of the amino terminus of the fibrinogen α-chain.[251]

Although *soluble fibrinogen* has been reported to associate specifically with β_2-integrin receptors on neutrophils under certain conditions,[281,291] it has been suggested that the affinity of these receptors for *soluble fibrinogen* is very low.[244,251] It has been suggested that clotted fibrinogen, or fibrinogen coated surfaces, in which the carboxyl terminus of the gamma chain is "multivalent", is the biologically relevant substrate for these receptors.[244,251] Neutrophils can also bind to soluble fibrinogen via a member of the β_3-integrin family, namely leukocyte response integrin (LRI).[240,254] Like the platelet β_3-integrin receptor, $\alpha_{IIb}\beta_3$, LRI reported to bind to both the R-G-D sequence of the fibrinogen α-chain as well as the K-Q-A-G-D-V sequence of the carboxyl terminus of the fibrinogen γ-chain. The interaction of neutrophils with soluble fibrinogen and fibrin(ogen) degradation products is discussed in greater detail in the following chapter (see pages 85-87).

References

1. Gallin JI. Disorders of phagocytic cells (1987) In *Harrison's Principals of Internal Medicine* (11th edition.) Braunwald E, Isselbacher KJ, Petersdorf RG, Wilson JD, Martin JB, Fauci AS. Eds. McGraw Hill, Hamburg. pp 290-292.
2. Borregaard N, Lollike K, Kjeldsen L. Sengelov H, Basholm L, Nielsen MH, Bainton DF. (1993) *Eur. J. Haematol.* **51**, 187.
3. Levy O. (1996) *Eur. J. Haematol.* **56**, 263.
4. Van der Valk P, Herman C. (1987) *Lab. Invest.* **57**, 127.
5. Cohen MS. (1994) *Clin. Infect. Dis.* **18**(Suppl. 2), S170.
6. Borregaard N. (1988) *Eur. J. Haematol.* **41**, 401.
7. Sieff CA. (1987) *J. Clin. Invest.* **79**, 1549.
8. Bainton DF, Ullyot JL, Farquar M. (1971) *J. Exp. Med.* **143**, 907.
9. Roitt J, Brostoff J, Male D. In: *Immunology*, 2nd Ed (1989) . Gower Medical Publishing, Churchill Livingstone, London.
10. Springer TA. (1990) *Nature (Lond.)*. **346**, 196.
11. Zimmerman GA, Prescott SM, Mc Intyre TM. (1992) *Immunol. Today*. **13**, 93.
12. Wardle EN. (1986) *Postgrad. Med. J.* **62**, 997.
13. Stossel TP. (1989) *J. Biol. Chem.* **264**, 18261.
14. Aderem A. (1992) *Trends Biochem. Sci.* **17**, 438.
15. Silverstein SC., Greenberg S, Di Virgilo F, Steinberg TH. (1989) In *Fundamental Immunology*. Paul WE Ed. Raven Press Ltd., New York. pp 703-719.
16. Geisow MJ, D'Arcy Hart P, Young MR. (1981) *J. Cell Biol.* **89**, 645.
17. Berger M, Wetzler E, August JT, Tartakoff AM. (1994) J. *Clin. Invest.* **94**, 1113.
18. Weiss SJ. (1989). *N. Engl. J. Med.* **320**, 365.
19. Gabay JE. (1994) *Antimicrobial Peptides. (Ciba Foundation Symposium)* **186**, 237.
20. Kuijpers TW, Tool ATJ, van der Schoot CE. (1991) *Blood.* **78**, 1105.
21. Miller LJ, Bainton DF, Borregaard N, Springer TA. (1987) *J. Clin. Invest.* **80**, 535.
22. Fletcher MP, Gallin JI. (1983) *Blood.* **62**, 792.
23. Singer II, Scott S, Kawka DW, Kazasis DM. (1989) *J. Cell Biol.* **109**, 3169.
24. Borregaard N, Heiple JM, Simons ER, Clarke RA. (1983) *J. Cell Biol.* **97**, 52.
25. Borregaard N, Cowland JB. (1997) *Blood.* **89**, 3503.
26. Borregaard N. (1996) *Curr. Opin. Haematol.* **3**, 11.
27. Le Cabec V, Cowland JB, Calafat J, Borregaard N. (1996) *Proc. Natl. Acad. Sci USA*. **93**, 6454.
28. Borregaard N, Sehested M, Nielsen BS, Sengelov H, Kjeldsen L. (1995) *Blood.* **85**, 812.
29. Lew PD, Wollheim C, Waldvogel FA, Pozzan T. (1984) *J. Cell Biol.* **99**, 1212.
30. Sengelov H, Kjeldsen L, Borregaard N. (1993) *J. Immunol.* **150**, 1535.
31. Sengelov H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, Borregaard N. (1995) *J. Immunol.* **154**, 4157.

32. Kjeldsen L, Bainton DF, Sengelov H, Borregaard. (1993) *Blood.* **82**, 3183.
33. Lollike K, Kjeldsen L, Sengelov H, Borregaard N. (1995) *Leukaemia.* **9**, 159.
34. Spicer SS, Hardin JH. (1969) *Lab. Invest.* **20**, 488.
35. Bainton DF, Farquar MG. (1966) *J. Cell Biol.* **28**, 277.
36. Cramer E, Pryzwansky KB, Villeval J-C, Testa U, Breton-Gorius J. (1985) *Blood.* **65**, 423.
37. Ohlsson K, Olsson I. (1974) *Eur. J. Biochem.* **42**, 519.
38. Bagglioni M. (1972) *Enzyme.* **12**, 132.
39. Csernok E, Ludemann J, Gross WL, Bainton DF. (1990) *Am. J. Pathol.* **137**, 1113.
40. Bretz U, Baglioni M. (1974) *J. Cell Biol.* **63**, 251.
41. Spitznagel JK, Dalldorf FG, Leffell MS, Folds JD, Welsh IRH, Cooney MH, Martin LE. (1974) *Lab. Invest.* **30**, 774.
42. Ganz T, Selsted M, Szklarek D, Harwig SSL, Daher K, Bainton DF, Lehrer RI. (1985) *J. Clin. Invest.* **76**, 1427.
43. Gabay JE, Scott RW, Campanelli D, Griffith J, Wilde C, Marra MN, Seeger M, Nathan CF. (1989). *Proc. Natl. Acad. Sci. USA.* **86**, 5610.
44. Weiss J, Olsson I. (1987) *Blood.* **69**, 652.
45. de Duve. (1975) *Science.* **189**, 186.
46. Dahlgren C, Carlssen SR, Karlssonn A, Lundqvist H, Sjolen C. (1995) *Biochem. J.* **311**, 667.
47. Cieutat AM, Lobel P, August JT, Kjeldsen L, Sengelov H, Borregaard N, Bainton DF (1998) *Blood.* **91**, 1044.
48. Cham BP, Gerrard JM, Bainton DF. (1994) *Am. J. Pathol.* **144**, 1369.
49. Saito N, Pulford KA, Breton-Gorius, J, Masse JM, Mason DY, Cramer EM. (1991) *Am. J. Pathol.* **139**, 1053.
50. Griffiths GM. (1996) *Trends Cell Biol.* **6**, 329.
51. Bangalore N, Travis J. (1994) *Biol. Chem. Hoppe-Seyler.* **375**, 659.
52. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. (1995) *J. Cell Biol.* **131**, 775.
53. Owen CA, Campbell MA, Boukedes SS, Campbell EJ. (1997) *Am. J. Physiol.* **272**, L385.
54. Gaudin P, Berthier S, Barro C, Zaoui P, Morel F. (1997) *Eur. J. Cell Biol.* **72**, 345.
55. Weersink AJL, van Kessel KPM, van den Tol ME. (1993) *J. Immunol.* **150**, 253.
56. Gullberg U, Anderson E, Garwicz D, Lindmark A, Olsson I. (1997) *Eur. J. Haematol.* **58**, 137.
57. Borregaard N, Tauber AI. (1984) *J. Biol. Chem.* **259**, 47.
58. Jesaitis A, Buescher ES, Harrison D, Quinn MT, Parkos CA, Livesey S, Linner J. (1990) *J. Clin Invest.* **85**, 821.
59. Sengelov H, Kjeldsen L, Diamond MS, Springer TA, Borregaard N. (1993) *J. Clin. Invest.* **92**, 1467.
60. Fletcher MP, Gallin JI. (1983) *Blood.* **62**, 792.
61. Jesaitis AJ, Naemara JR, Painter RG, Sklar LA, Cochrane CG. (1982) *Biochim. Biophys. Acta.* **719**, 556.
62. Porteu F, Nathan CF. (1992) *J. Leuk. Biol.* **52**, 122.
63. Murphy G, Bretz U, Baglioni M, Reynolds JJ. (1980) *Biochem. J.* **192**, 517.

64. Santibanez JF, Martinez J. (1993) *Leuk. Res.* **17**, 1057.
65. Heipel JM, Ossowski L. (1986) *J. Exp. Med.* **164**, 826.
66. Kane SP, Peters TJ. (1975) *Clin. Sci. Mol. Med.* **49**, 171.
67. Kjeldsen L, Bjerrum OW, Askaa J, Borregaard M. (1992) *Biochem. J.* **287**, 603.
68. Kjeldsen L, Sengelov H, Lollike K, Nielsen MH, Borregaard N. (1994) *Blood.* **83**, 1640.
69. Murphy G, Ward R, Hembry RM, Reynolds JJ, Kuhn K, Tryggvason K. (1989) *Biochem J.* **258**, 463.
70. Hibbs MS, Hasty KA, Seyer JM, Kang AH, Mainardi CL. (1985) *J. Biol. Chem.* **260**, 2493.
71. Murphy G, Reynolds JJ, Bretz U, Bagglioni M. (1982) *Biochem. J.* **203**, 209.
72. Delclaux C, Delacourt C, d'Ortho MP, Boyer V, Lafuma C, Harf A. (1996) *Am. J. Resp. Cell. Mol. Biol.* **14**, 288.
73. Leppert D, Waubant E, Galardy R, Bunnett NW, Hauser SL. (1995) *J. Immunol.* **154**, 4379.
74. Borregaard N, Christiansen L, Bjerrum OW, Birgens Hs, Clemmenson I. (1990) *J. Clin Invest.* **85**, 408.
75. Borregaard N, Miller L, Springer TA. (1987) *Science* **237**, 1204.
76. Sengelov H, Nielsen MH, Borregaard N. (1992) *J. Biol. Chem.* **267**, 14912.
77. Calafat J, Kuijpers T, Jansen H, Borregaard N. (1993) *Blood.* **81**, 3122.
78. Sengelov H, Boulay F, Kjeldsen L, Borregaard N. (1994) *Biochem. J.* **299**, 473.
79. Plesner T, Plough M, Ellis V, Ronne E, Hoyer-Hansen G, Wittrup M, Pedersen TL, Tscherning T, Dano K, Hansen NE. (1994) *Blood.* **83**, 808.
80. Sengelov H, Kjeldsen L, Kroese W, Berger M, Borregaard N. (1994) *J. Immunol.* **153**, 804.
81. Borregaard N, Kjeldsen L, Rygaard K, Bastholm L, Nielsen MH, Sengelov H, Bjerum OW, Johnsen AH. (1992) *J. Clin. Invest.* **90**, 86.
82. Kobayashi T, Robinson JM. (1991) *J. Cell Biol.* **113**, 743.
83. Fernandez-Segura E, Garcia JM, Campos A. (1995) Histochem. *Cell Biol.* **104**, 175.
84. Waddell TK, Fialkow L, Chan CK, Kishimoto TK, Downey GP. (1994) *J. Biol. Chem.* **269**, 18485.
85. Crocket-Torabi E, Fantone JC. (1995) *Immunol. Res.* **14**, 237.
86. Jeannin P, Delneste Y, Gosset P, Molet S, Lasalle P, Hamid Q, Tsicopoulos A, Tonnel AB. (1994) *Blood.* **84**, 2229.
87. Patel KD, Modur V, Zimmerman GA, Prescott SM, Mc Intyre TM. (1994) *J. Clin. Invest.* **94**, 631.
88. Hughes BJ, Hollers HC, Crocket-Torabi E, Smith CW. (1992) *J. Clin. Invest.* **90**, 1687.
89. Borregaard N, Kjeldsen L, Sengelov H, Diamond MS, Springer TA, Anderson HC, Bainton DF, Kishimoto TK. (1994) J. *Leuk. Biol.* **56**, 80.
90. Borregaard N, Kjeldsen L, Lollike K, Sengelov H. (1993) *Eur. J. Haematol.* **51**, 318.
91. Gabay JE, Almeda RP. (1993) *Curr. Opin. Immunol.* **5**, 97.
92. Floodgaard H, Ostergaard E, Bayne S. (1991) *Eur. J. Biochem.* **197**, 535.
93. Morgan JG, Sukiennicki T, Pereira HA, Spitznagel JK, Guerra ME, Larrick JW. (1991) *J. Immunol.* **147**, 3210.

94. Galli SJ, Hammel I. (1994) *Curr. Opin. Haematol.* **1**, 33.
95. Zimmer M, Metcalf RL, Fink TM, Mattman C, Lichter P, Jenne DE (1992) *Proc. Natl. Acad. Sci. USA*. **89**, 8215.
96. Hanson RD, Hohn PA, Popescu NC, Ley TJ. (1990) *Proc. Natl. Acad. Sci. USA*. **87**, 960.
97. Caughey GH. (1994) *Am. J. Resp. Crit. Care Med.* **150**, s138.
98. Gullberg U, Lindmark A, Lindgren G, Persson AM, Nilsson E, Olsson I. (1995) *J. Cell Biol.* **270**, 12912.
99. Campbell EJ, Senior RM, Welgus HG. (1987) *Chest.* **92**, 161.
100. Janoff A. (1985) *Am. Rev. Respir. Dis.* **132**, 417.
101. Barrett AJ. (1985) *Agents Actions.* **8**, 11.
102. Janoff A, Feldstein G, Malemud CJ, Elias JM. (1976) *J. Lab. Clin. Invest.* **57**, 615.
103. MalechHL, Gallin JI. (1987) *N. Engl. J. Med.* **317**, 687.
104. Sinha S, Watorek W, Karr S, Giles J, Bode W, Travis J. (1987) *Proc. Natl. Acad. Sci.* **84**, 2228.
105. Bode W, Wei A, Huber R, Meyer E, Travis J, Neumann S. (1986) *EMBO J.* **5**, 2453.
106. Janoff A. (1985) *Ann. Rev. Med.* **36**, 207
107. Mc Donald JA, Kelly DG. (1980) *J. Biol. Chem.* **25**, 8848.
108. Plow EF. (1980) *Biochim. Biophys. Acta.* **630**, 47.
109. Cepinskas G, Noseworthy R, Kvietys PR. (1997) *Circ. Res.* **81**, 618.
110. Zimmerman BJ, Granger DN. (1990) *Am. J. Physiol.* **259**, H390.
111. Woodman RC, Heinhart PH, Kanwar S, Johnston FL, Kubes P. (1993) *Blood.* **82**, 2188.
112. Carden DL, Korthuis RJ. (1996) *Am. J. Physiol.* **271**, H1947.
113. Cai T-Q, Wright SD. (1996) *J. Exp. Med.* **184**, 1213.
114. Bedard M, Mc Clure CD, Schiller NL, Francoeur C, Cantin A, Denis M. (1993) *Am. J. Resp. Cell. Mol. Biol.* **9**, 455.
115. Nakamura H, Yoshimura K, Mc Elvaney NG, Crystal RG. (1992) *J. Clin. Invest.* **89**, 1478.
116. Ruef C, Jefferson DM, Schlegel-Haueter SE, Suter S. (1993) *Eur. Resp. J.* **6**, 1429.
117. Banda M, Rice JAG, Criffin GL, Senior RM. (1988) *J. Biol. Chem.* **263**, 4481.
118. Hubbard RC, Fells G, Gadek J, Pachalok S, Humes J, Crystal RG. (1991) *J. Clin. Invest.* **88**, 891.
119. Kusner DJ, King CH. (1989) *J. Immunol.* **143**, 1696.
120. Odeberg H, Olsson I. (1976) *Infect. Immun.* **14**, 269.
121. Thorne KJI, Oliver CR, Barrett AJ. (1976) *Infect. Immun.* **14**, 555.
122. Belaauoaj A, Mc Carthy R, Baumann M, Gao Z, Ley T, Abraham SN, Shapiro SD. (1998) *Nature Medicine.* **4**, 615.
123. Speer CP, Pabst MJ, Hedegaard HB, Rest RF, Johnston RB. (1984) *J. Immunol.* **133**, 2151.
124. Si-Tahar M, Pidard D, Balloy V, Moniatte M, Kieffer N, van Dorsselaer A, Chignard M. (1997) *J. Biol. Chem.* **272**, 11636.
125. Vischer TL, Bretz U, Bagglioni M. (1976) *J. Exp. Med.* **144**, 863.

126. Bussolino F, Arese M, Silvestro R, Soldi R, Benfenati E, Sanavio F, Aglietta M, Bosia A, Camussi G. (1994) *Eur. Resp. J.* **24**, 3131.
127. Porteu F, Brockhaus M, Wallach D, Engelmann H, Nathan CF. (1991) *J. Biol. Chem.* **266**, 18846.
128. Champagne B, Tremblay, P Cantin A, St. Pierre Y. (1998) *J. Immunol.* **161**, 6398.
129. Yamagushi Y, Matsumura F, Wang F-s, Akazuki E, Liang J, Matsuda T, Okabe K, Ohshiro H, Horiuchi T, Yamada S, Mori K, Ogawa M. (1998) *Transplantation.* **65**, 1622.
130. Ariel A, Yavin EJ, Hershkoviz R, Avron A, Franitza S, Hardan I, Cahalon L, Fridkin M, Lider O. (1998) *J. Immunol.* **161**, 2465.
131. Padrines M, Wolf M, Walz A, Bagglioni M. (1994) *FEBS Letters.* **352**, 231.
132. Scuderi P. Nez PA, Duerr ML, Wong BJ, Valdez CM. (1991) *Cell. Immunol.* **135**, 299.
133. Van Kessel KPM, Van Strijp JAG, Verhoef J. (1991) *J. Immunol.* **147**, 3862.
134. Orr FW, Varani J, Kreutzer DL, Senior RM, Ward PA. (1979) *Am. J. Pathol.* **94**, 75.
135. Taylor JC, Crawford IP, Hugli TE. (1977) *Biochemistry.* **16**, 3390.
136. Henson PM. (1971) *J. Immunol.* **107**, 1547.
137. Travis J, Salvesen GS. (1983) *Ann. Rev. Biochem.* **52**, 655.
138. Kramps JA, Rudolphus A, Stolk J, Willems LNA, Dijkman JH. (1991) *Ann. N.Y. Acad. Sci.* **624**, 97.
139. Campbell EJ, Campbell MA. (1988) *J. Cell Biol.* **106**, 667.
140. Campbell EJ, Silverman EK, Campbell MA. (1989) *J. Immunol.* **143**, 2961.
141. Rice WG, Weiss SJ. (1990) *Science.* **249**, 178.
142. Weiss SJ, Regiani S. (1984) *J. Clin. Invest.* **73**, 1297.
143. Clark RA, Stone PJ, Hag AE, Calore JD, Franzblau C. (1981) *J. Biol. Chem.* **256**, 3348.
144. Vissers MCM, Geroge PM, Bathurst CI, Brennan SU, Winterbourn, CC. (1988) *J. Clin. Invest.* 82, **706**.
145. Banda MJ, Clark EJ, Sinha S, Travis J. (1987) *J. Clin. Invest.* **79**, 1314.
146. Campbell EJ, Senior RM, Mc Donald JA, Cox DL. (1982) *J. Clin. Invest.* **70**, 845.
147. Weitz JI, Crowley KA, Landman SL, Lipman BI, Yu J. (1987) *Ann. Internal Med.* **107**, 680.
148. Loike JD, Silverstein R, Wright SD, Weitz JI, Huang AJ, Silverstein SC. (1992) *Ann. NY. Acad. Sci.* **667**, 163.
149. Chapman HA Jnr., Vavrin Z, Hibbs JB. (1982) *Cell.* **28**, 653.
150. Plow EF, Freaney DE, Plescia J, Miles LA. (1986) *J. Cell Biol.* **103**, 2411.
151. Dwenger A, Tost P, Holle W. (1986) *J. Clin. Chem. Clin. Biochem.* **24**, 299.
152. Travis J. (1988) *Am. J. Med.* **84** (suppl.), 37.
153. Kolev K, Komorowicz E, Owen WG, Machovich R. (1996) *Thromb. Haemostas.* **75**, 140.
154. Thomas RN, Nauseef WM, Iyer SS, Petersen MW, Stone PJ, Clark RA. (1991) *J. Leuk. Biol.* **50**, 568.
155. Venge P, Olsson I. (1975) *J. Immunol.* **115**, 1505.
156. Baici A, Knopfel M, Fehr K. (1982) *Mol. Immunol.* **19**, 719.

157. Wintroub BU, Coblyn JS, Kaempfer CE, Austen KF. (1980) *Proc. Natl. Acad. Sci. USA.* **77**, 5448.
158. Vartio T, Seppa H, Vaheri A. (1981) *J. Biol. Chem.* **256**, 471.
159. Chertov O, Ueda H, Xu LL, Tani K, Murphy WJ, Wang JM, Howard OMZ, Sayers TJ, Oppenheim JJ. (1997) *J. Exp. Med.* **186**, 739.
160. Kubes P, Smith P, Grisham MD, Granger DN. (1993) *J. Inflamm.* **17**, 321.
161. Ferrer-Lopez P, Renesto P, Schattner M, Bassot S, Laurent P, Chignard M. (1990) *Am. J. Physiol.* **258**, C1100.
162. Renesto P, Chignard M. (1991) *J. Immunol.* **146**, 2305.
163. Renesto P, Chignard M. (1993) *Blood.* **82**, 139.
164. Yavin EJ, Yan L, Desiderio DM, Fridkin M. (1996) *Int. J. Pept. Protein Res.* **48**, 465.
165. Yavin EJ, Fridkin M. (1998) *J. Pept. Res.* **51**, 282.
166. Goldschmeding R, van der Schoot CE, ten Bokkel Huinink D, Hack CE, van den Ende ME, Kallenberg CGM, von dem Borne AEG. (1989) *J. Clin. Invest.* **84**, 1577.
167. Ohlsson K, Linder C, Rosengren M. (1990) *Biol. Chem. Hoppe-Seyler.* **371**, 549.
168. Rao NV, Wehner NG, Marshall BC, Gray WR, Gray BH, Hoidal JR. (1991) *J. Biol. Chem.* **266**, 9540.
169. Wilde CG, Snable JL, Griffith JE, Scott RW. (1990) *J. Biol. Chem.* **265**, 2038.
170. Campanelli D, Detmer PA, Nathan CF, Gabay JE. (1990) *J. Clin. Invest.* **85**, 905.
171. Renesto P, Halbwachs-Mecarelli, Nusbaum P, Lesavre P, Chignard M. (1994) *J. Immunol.* **152**, 4612.
172. Csernok, Ernst M, Scmitt W, Bainton DF, Gross WL. (1994) *Clin. Exp. Immunol.* **95**, 244.
173. van der Woude FJ, Rasmussen N, Labatto S. (1985) *Lancet.* **I**, 425.
174. Jennings JG, Chang L, Savige JA. (1994) *Clin. Exp. Immunol.* **95**, 251.
175. Zimmer M, Medcalf RL, Fink TM, Mattaman C, Lichter P, Jenne DE. (1992) *Proc. Natl. Acad. Sci. USA.* **89**, 8215.
176. Wilde CG, Snable JL, Griffith JE, Scott RW. (1990) *J. Biol. Chem.* **265**, 2038.
177. Pereira AH, Shafer WM, Pohl J, Martin LE. (1990) *J. Clin. Invest.* **85**, 1468.
178. Hirose T, Patterson C, Pourmotabbed T, Mainardi Cl, Hasty KA. (1993) *Proc. Natl. Acad. Sci. USA.* **90**, 2569.
179. Bu CH, Pourmotabbed T. (1995) *J. Biol. Chem.* **270**, 18563.
180. Woesner JF. (1991) *FASEB J.* **5**, 2145.
181. Birkedal-Hansen H, Moore WGI, Bodden MK, Windsor LJ, Birkedal-Hansen B, De Carlo A, Engler JA. (1993) *Crit. Rev. Oral Biol. Med.* **4**, 197.
182. Matrisan LM. (1992) *Bioessays.* **14**, 455.
183. Sato H, Takino T, Okada Y, CaoJ, Shinagawa A, Yamamoto E, Seili M. (1994) *Nature.* **370**, 61.
184. Rice A, Banda MJ. (1995) *Biochemistry.* **34**, 9249.
185. Matirisian LM. (1990) *Trends Genet.* **6**, 121.
186. Bakowski B, Tschetsche H. (1992) *Biol. Chem. Hoppe Seyler.* **373**, 529.
187. Knauper V, Kramer S, Reinke H, Tschetsche H. (1990) *Eur. J. Biochem.* **189**, 295.

188. Murphy G, Cocket MI, Stephens PE, Smith BJ, Docherty AJ. (1987) *Biochem. J.* **248**, 265.
189. Hasty KA, Hibbs MS, Kang AH, Mainardi CL. (1986) *J. Biol. Chem.* **261**, 5645.
190. Pontremoli S, Melloni E, Michetti M, Sacco O, Sparatore B, Salamino F, Damiani G, Horecker BL. (1986) *Proc. Natl. Acad. Sci. USA.* **83**, 1685
191. Melloni E, Pontremoli S, Salamino F, Sparatore B, Michetti M, Sacco O, Horecker BL. (1986) *J. Biol. Chem.* **261**, 11437.
192. Pontremoli S, Melloni E, Sparatore B, Michetti M, Salamino F. (1986) *J. Biol. Chem.* **265**, 706.
193. King CH, Goralnik CH, Kleinhenz PJ, Marino JA, Sedor JR, Mahmoud AAF. (1987) *J. Clin. Invest.* **79**, 1091.
194. King CH, Hull A, Kleinhenz PJ, Phillips NFB, Marino JA. (1991) *J. Immunol.* **146**, 3115.
195. Malech HL, Gardner JP, Heiman DF, Rosenzweig SA. (1985) *J. Biol. Chem.* **260**, 2509.
196. Allen RA, Tolley JO, Jesaitis JA. (1986) *Biochim. Biophys. Acta.* **882**, 271.
197. Shephard EG, Kelly Sl, Anderson R, Fridkin M. (1992) *Clin. Exp. Immunol.* **87**, 509.
198. Kelly SL, Adams SA, Robson SC, Kirsch RE, Shephard EG. (1994) *Biochem. J.* **298**, 689.
199. Adams SA, Kelly SL, Kirsch RE, Robson SC, Shephard EG. (1995) *Blood Coag. Fibrinol.* **6**, 693.
200. Longhurst CM, Jennings LK. (1998) *Cell. Mol. Life Sci.* **54**, 514.
201. Clemetson KJ. (1998) *Cell. Mol. Life Sci.* **54**, 499.
202. Ruoslahti E, Pierschbacher MD. (1987) *Science.* **238**, 491.
203. Buck CA. (1987) *Annu. Rev. Cell. Biol.* **3**, 179.
204. Miyamoto S, Teramoto H, Coso O, Gutkind JS, Burbelo PD, Akayima SK, Yamada KM. (1995) *J. Cell. Biol.* **131**, 791.
205. Gresham HD, Goodwin JL, Allen PM, Anderson DC, Brown EJ. (1989) **108**, 1935.
206. Pakianathan DR. (1995) *J. Leuk. Biol.* **57**, 699.
207. Nathan C. (1987) *J. Clin. Invest.* **80**, 1550.
208. Nathan C, Srimal S, Farber C, Sanchez, Kabash L, Asch A, Gailit J, Wright SD. (1989) *J. Cell Biol.* **109**, 1341.
209. O'Toole TE, Loftus JW, Plow EF, Glass AA, Harper JR, Ginsberg MH. (1989) *Blood.* **74**, 14.
210. Smythe SS, Joneckis CC, Parise LV. (1993) *Blood.* **81**, 2827.
211. Brown EJ. (1997) *Semin. Haematol.* **34**, 319.
212. Ginsberg MH, Loftus JC, Plow EF. (1988) *Thromb. Haemostas.* **59**, 1.
213. Kieffer N, Phillips DR. (1990) *Annu. Rev. Cell Biol.* **6**, 329.
214. Pavalko FM, Otey CA. (1994) *Proc. Soc. Exp. Biol. Med.* **205**, 282.
215. Parise LV, Phillips DR. (1986) *J. Biol. Chem.* **261**, 14011.
216. Marlin SD, Springer TA. (1987) *Cell.* **51**, 813.
217. Bennett JS, Vilaire G. (1979) *J. Clin Invest.* **64**, 1393.
218. Hemler ME. (1990) *Annu. Rev. Immunol.* **8**, 365.
219. Ruoslahti E. (1991) *J. Clin. Invest.* **87**, 1.

220. Gahmberg CG, Valmu L, Fagerholm S, Kotovouri P, Ihanus E, Tian L, Pessa-Morikawa T. (1998) *Cell. Mol. Life Sci.* **54**, 549.
221. Dickeson SK, Santoro SA. (1998)) *Cell. Mol. Life Sci.* **54**, 556.
222. Gailit J, Rouslahti E. (1998) *J. Biol. Chem.* **263**, 12927.
223. Steiner B, Cousot D, Trzeciak A, Gillessen D, Hadvary P. (1989) *J. Biol. Chem.* **264**, 13102.
224. Ginsberg MH, Lightsey A, Kunicki TJ, Kaufman A, Marguerie GA, Plow EF. (1986) *J. Clin. Invest.* **78**, 1103.
225. Diamond MS, Springer TA. (1993) *J. Cell Biol.* **120**, 545.
226. Anderson DC, Springer TA. (1987) *Annu. Rev. Med.* **38**, 175.
227. Van der Vieren M, Trong HL, Wood CL, Moore PF, St. John T, Staunton DE. (1995) *Immunity.* **3**, 683.
228. Arnaout MA (1990) *Immunol. Rev.* **114**, 145.
229. Diamond MS, Garcia-Aguilar J, Bickford JK, Corbi AL, Springer TA. (1993) *J. Cell Biol.* **120**, 1031.
230. O'Toole TE, Katagiri Y, Faull RJ, Peter K, Tamura R, Quaranta V. (1994) *J. Cell Biol.* **124**, 1047.
231. Andrew D, Shock A, Ball E, Ortlepp S, Bell J, Robinson M. (1993) *Eur. J. Immunol.* **23**, 2217.
232. Davignon D, Marte E, Reynolds T, Kurzinger K, Springer TA. (1981) *Proc. Natl. Acad. Sci. USA.* **78**, 4535.
233. Schnitzler N, Haase G, Podbielski A, Lutticken R, Schweizer KG. (1999) *Nature Medicine.* **5**, 231.
234. Smith CW, Marlin SD, Rothlein R, Toman C, Anderson DC. (1989) *J. Clin. Invest.* **83**, 2008.
235. Adams DH, Shaw S. (1994) *Lancet.* **343**, 831.
236. Anderson DC, Miller LJ, Schmalstieg FC, Rothlein R, Springer TA. (1986) *J. Immunol.* **137**, 15.
237. Patarroyo MP, Beatty PG, Serhan CN, Gahmberg CG. (1985) *Scand. J. Immunol.* **22**, 619.
238. Wright SD, Silverstein SC. (1983) *J. Exp. Med.* **158**, 2016.
239. Wright SD, Meyer BC. (1986) *J. Immunol.* **136**, 1759.
240. Graham IL, Gresham HD, Brown EJ. (1989) *J. Immunol.* **142**, 2352.
241. Beller DI, Springer TA, Shreiber RD. (1982) *J. Exp. Med.* **156**, 1000.
242. Mosser DM, Springer TA, Diamond MS. (1992) *J. Cell Biol.* **116**, 511.
243. Altieri DC, Bader R, Mannucci PM, Edgington TS. (1988) *J. Cell Biol.* **107**, 1893.
244. Wright SD, Weitz JI, Huang AJ, Levin SM, Silverstein SC, Loike JD. (1988) *Proc. Natl. Acad. Sci. USA.* **85**, 7734.
245. Altieri DC, Edgington TS. (1988) *J. Biol. Chem.* **263**, 7007.
246. Simon SI, Chambers JD, Butcher E, Sklar LA. (1992) *J. Immunol.* **149**, 3557.
247. Lanier LL, Arnaout MA, Schwarting P, Warner NL, Ross GD. (1985) *Eur. J. Immunol.* **15**, 713.
248. Miller LJ, Schwarting R, Springer TA. (1986) *J. Immunol.* **137**, 2891.
249. Springer TA, Miller LJ, Anderson DC. (1986) *J. Immunol.* **136**, 240.
250. Stacker SA, Springer TA. (1991) *J. Immunol.* **146**, 648.

251. Loike JD, Sodeik B, Cao L, Leucona S, Weitz JI, Detmers PA, Wright SD, Silverstein SC. (1991) *Proc. Natl. Acad. Sci. USA.* **88**, 1044.
252. Bilsland CA, Diamond MS, Springer TA. (1994) *J. Immunol.* **152**, 4582.
253. Ingalls RR, Golenbock DT. (1995) *J. Exp. Med.* **181**, 1473.
254. Gresham HD, Adams SP, Brown EJ. (1992) *J. Biol. Chem.* **267**, 13895.
255. Brown E, Hooper L, Ho T, Gresham H. (1990) *J. Cell Biol.* **111**, 2785.
256. Zhou M, Brown EJ. (1993) *J. Exp. Med.* **178**, 1165.
257. Senior RM, Gresham HD, Griffin CL, Brown EJ, Chung AE. (1992) *J. Clin. Invest.* **90**, 2251.
258. Mc Ever RP. (1991) *Thromb. Haemostas.* **65**, 223.
259. Bevilacqua MP. (1993) *Annu. Rev. Immunol.* **11**, 767.
260. Kansas GS, Spertini O, Stoolman LM, Tedder TF. (1991) *J. Cell Biol.* **114**, 315.
261. Lawrence MB, Springer TA. (1991) *Cell.* **65**, 859.
262. Lasky LA. (1992) *Science.* 258, 964.
263. Kuijpers TW, Hakkert BC, Hoogerwerf M, Leuwenberg JFM, Roos D. (1991) *J. Immunol.* **147**, 1369.
264. Lo SK, Lee S, Ramos RA, Lobb R, Rosa M, Chi-Rosso G, Wright SD. (1991) *J. Exp. Med.* **173**, 1493.
265. Simon SI, Burns AR, Taylor AD, Gopalan PK, Lynam EB, Sklar LA, Wayne-Smith C. (1995) *J. Immunol.* **155**, 1502.
266. Spertini O, Kansas GS, Munro JM, Griffin JD, Tedder TF. (1991) *Nature.* **349**, 691.
267. Kishimoto TK, Jutila MA, Berg EL, Butcher EC. (1989) *Science.* **245**, 1238.
268. Bevilacqua MP, Stengelin S, Gimbrone MA Jnr, Seed B. (1989) *Science.* **243**, 1160.
269. Lorant DE, Patel KP, Mc Intyre TM, Mc Ever RP, Prescott SM, Zimmerman GA. (1991) *J. Cell Biol.* 115, 223.
270. Hattori R, Hamilton KK, Fugate RD, Mc Ever RP, Sims PJ. (1989) *J. Biol. Chem.* **264**, 7768.
271. Springer TA. (1990) *Nature.* **346**, 425.
272. Staunton DE, Dustin ML, Springer TA. (1989) *Nature.* **339**, 61.
273. Staunton DE, Dustin ML, Erikson HP, Springer TA. (1990) *Cell.* **61**, 243.
274. Diamond MS, Staunton DE, de Fougerolles AR. (1990) *J. Cell Biol.* **111**, 3129.
275. Barnett CC, Moore EE, Mierau GW, Partrick DA, Biffl WL, Elzi DJ, Silliman CC. (1998) *Am. J. Physiol.* **274**, C1634.
276. Rot A. (1992) *Immunol. Today.* **13**, 291.
277. Hubere AR, Kunkel SL, Todd RF III, Weiss SJ. (1991) *Science.* **254**, 99.
278. Hibbs ML, Xu H, Stacker SA, Springer TA. (1991) *Science.* **251**, 1611.
279. Hibbs ML, Jakes S, Stacker SA. (1991) *J. Exp. Med.* **174**, 1227.
280. Carlos TM, Harlan JM. (1990) *Immunol. Rev.* **114**, 5.
281. Altieri D, Plescia J, Plow EF. (1993) *J. Biol. Chem.* **268**, 1847.
282. Laudanna C, Miron S, Berton G, Rossi F (1990) *Biochem. Biophys. Res. Commun.* **166**, 308.
283. Laudanna C, Miron S, Berton G, Rossi F (1993) *Biochem. Biophys. Res. Commun.* **190**, 935.
284. Nathan CF, Sanchez E. (1990) *J. Cell Biol.* **111**, 2171.
285. Fuortes M, Jin WW, Nathan C. (1993) *J. Cell Biol.* **120**, 777.

286. Berton G, Fumagalli L, Laudanna C, Sorio C. (1994) *J. Cell Biol.* **126**, 1111.
287. Gomez-Cambronero J, Huang C-H, Bonak VA, Wang E, Casnellie J-E, Shiraishi T, Sha'afi RI. (1989) *Biochem. Biophys. Res. Commun.* **162**, 1478.
288. Naccahe PH, Gilbert C, Caon AC, Gaudry M, Huang C-K, Bonak VA, Umezawa K, Mc Coll SR. (1990) *Blood.* **76**, 2098.
289. Kusunoki T, Higashi H, Hosoi S, Hata D, Sugie K, Mayumi M, Mikawa H. (1992) *Biochem. Biophys. Res. Commun.* **183**, 789.
290. Succhard SJ, Boxer LA. (1994) *J. Immunol.* **152**, 290.
291. Ugarova TP, Solovjov DA, Zhang L, Loukinov DI, Yee VC, Medved LV, Plow EF (1998*) J. Cell Biol.* **273**, 22519.

Chapter Three

Fibrinogen

Fibrinogen is best known for its role in haemostasis in which it serves as a precursor for fibrin clots and an essential ingredient of platelet aggregates. In addition to its role in haemostasis, fibrinogen has become increasingly recognised as a multifunctional protein which interacts with a variety of cells and proteins, and regulates a number of important biological processes.

Structure

Fibrinogen is a 340 kDa acid glycoprotein, comprising approximately 3000 amino acids, and made up of two identical halves.[1,2] Each half molecule contains 3 distinct polypeptide chains, known as the Aα, Bβ and γ chains,* and these two halves are held together by a network of disulphide bonds[3-5] (figure 1).

Structural studies have revealed fibrinogen to be an elongated, trinodal molecule, with its six polypeptide chains arranged in three globular domains, one located centrally (known as the E-domain) and two peripherally (the D-domains).[6-8] The central E domain is formed by the amino termini of all six chains as they come together at the dimer interface, whereas the two peripheral D domains are formed by the carboxyl termini of the beta and gamma chains (figure 1).[9-11] The alpha chain carboxyl termini do not form part of the D-domain, but are thought to fold back and interact directly with the central globular domain.[12-14] The E domain is linked to the D domains by a triple helix rope-like connector, comprising a 111 amino acid residue stretch of the 3 polypeptide chains, twisted tightly round one another and interacting via hydrogen bonds, to form a three stranded helix frequently referred to as the "coiled coil."[15]

* Also referred to as the α, β and γ chains.

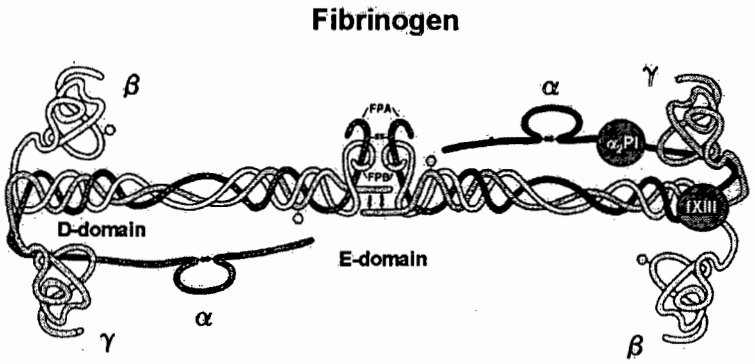


Figure 1. A schematic representation of the fibrinogen molecule. *Fibrinogen consists of two half molecules, each comprising three polypeptide chains (Aα, Bβ and γ). These half molecules are joined by disulphide bonds at the amino terminal regions of the 3 polypeptide chains. This arrangement gives fibrinogen a trinodal structure with the central E-domain comprising the amino termini of the six polypeptide chains, and the peripheral D-domains comprising the carboxyl termini of the Bβ and γ chains. The alpha chain carboxyl termini fold back and interact directly with the central globular domain. The E- and D-domains are linked by a triple helix formed by the 3 polypeptide chains, frequently referred to as the "coiled coil." Also indicated are the factor XIII (fXIII) and (α_2PI) α_2-antiplasmin binding sites. (Adapted from Reference 100)*

The fibrinogen molecule is stabilised by 29 disulphide bonds, 17 of which link the 6 chains to one another (*interchain* disulphide bonds), and 12 of which link different regions within individual polypeptide chains (*intrachain* disulphide bonds).[16-19]
The assembly of the two half molecules is dependent upon 3 *interchain* disulphide bonds at the amino terminus as well as three pairs in the amino terminal region of the coiled coil.[20-22] In contrast, the interchain disulphide linkages on the carboxyl terminal side of the coiled coil region are not essential for chain assembly; however, if they are not present fibrinogen is assembled but not secreted, probably due to improper folding.[20]

Each half molecule has six *intrachain* disulphide bonds, which form loops in the carboxyl-terminal regions of each polypeptide chain; the Aα chain has one, the Bβ chain three, and the γ chain has two such bonds.[23] This combination of inter- and intrachain disulphide linkages together with the coiling of the three chains, imparts a

specific structure to fibrinogen which is likely to influence its biological activities.

Biosynthesis and regulation

The fibrinogen gene

Each polypeptide chain is encoded by a separate mRNA, transcribed from 3 distinct, single copy genes.[24-26] The cDNAs for each chain of human fibrinogen have been cloned and their nucleotide sequences are known.[26-31] The striking homology that exists between the cDNAs encoding the three chains suggests that they may have evolved from a common gene. Furthermore, the observation, in both the rat and the human, that the α and γ genes lie in an opposite transcriptional orientation to the β gene, and that all three lie in very close proximity to one another, suggests that both gene duplication and inversion may have been involved in the evolution of the three chains of fibrinogen from a single ancestral gene.[24,25]

Fibrinogen biosynthesis

Fibrinogen is synthesised by the hepatocyte[32] at a rate of 1.7-5 grams per day under steady state conditions.[33,34] Once in the circulation, fibrinogen has a half-life of approximately 3-5 days.[35]

During the steady state, the synthesis of the Bβ chain appears to be the important rate limiting step in fibrinogen synthesis,[36,37] whereas during the acute phase response (see below), a proportional increase in the mRNA encoding all three chains is seen.[38] Following synthesis on separate polysomes, the three translated chains are transported to an assembly site within the lumen of the endoplasmic reticulum where they are assembled by electrostatic, hydrophobic and disulphide interactions into a dimeric structure.[39] This involves a pathway of sequential steps with the generation of a number of probable intermediates. Although there are conflicting reports as to

the nature of these intermediates,[36,40-47] the final step of this pathway appears to involve the dimerisation of two AαBβγ half molecules, with disulphide bonds in the amino terminal region of the Aα and γ chains joining these two half molecules together (figure 2).[40-42]

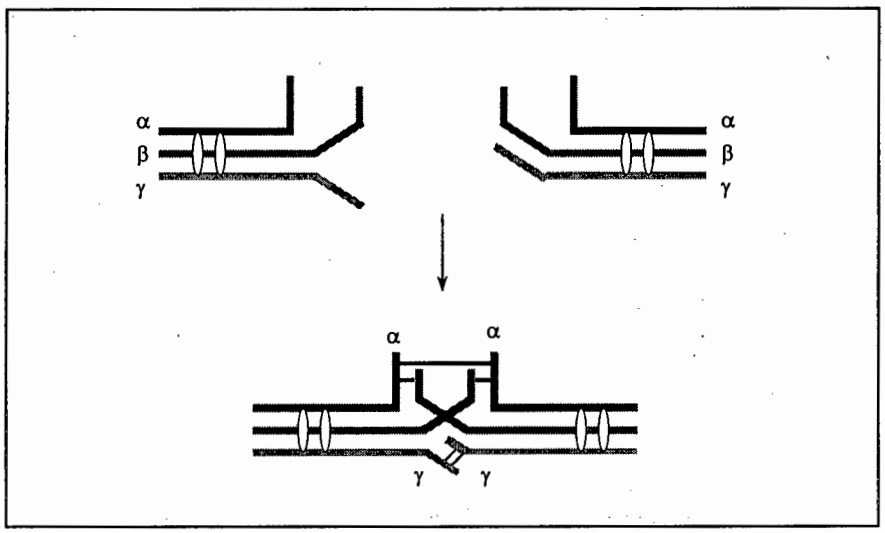


Figure 2. *A schematic representation of the final step in fibrinogen assembly which involves the dimerisation of the two AαBβγ half molecules. Disulphide bonds in the amino terminal region of the Aα and γ chains join the two half molecules together.*

Regulation of fibrinogen synthesis

In addition to the opposing forces of synthesis and catabolism that regulate the levels of any plasma protein, fibrinogen levels in the plasma are influenced by two additional factors. Firstly, fibrinogen (either soluble or clotted) is also degraded by plasmin, the protease responsible for the dissolution of fibrin clots. Secondly, a variety of inflammatory conditions (including infection, physical, chemical or thermal trauma, and neoplasia) may provoke a systemic response associated with changes in the plasma levels of a group of proteins (known as acute phase reactants) of which fibrinogen is one.[48] During this response, known as the acute phase response, the concentration of fibrinogen may increase 2-20-fold, and is associated with a similar elevation in mRNA levels.[49-51] Thus, in determining

the factors which regulate fibrinogen synthesis, it is important to distinguish between *non-specific* stimuli, which induce fibrinogen synthesis through activation of the acute phase response, and *specific* signals for fibrinogen synthesis.

Several non-specific stimuli of fibrinogen synthesis have been identified. Peripheral blood leukocytes, when incubated with plasmin derived fragments of fibrinogen or fibrin, have been reported to secrete a factor which upregulates the synthesis of fibrinogen (and other acute phase proteins) by rat hepatocytes.[52] Similarly, human peripheral blood monocytes have been shown to release a factor which is capable of inducing increased production of acute phase proteins by cultured hepatocytes.[53-55] This factor was subsequently identified as interleukin 6.[56-58] On the other hand, several factors, including interleukin-1 (IL-1), tumour necrosis factor (TNF)[57] and transforming growth factor β (TGFβ),[59] have been shown exert an inhibitory effect on hepatocyte production of acute phase proteins.

The possibility that fibrinogen levels may serve as a feedback mechanism for fibrinogen synthesis has been considered. Indeed, a massive defibrination in rats (effected by the administration of Malayan pit viper venom) was shown to induce an increase in fibrinogen mRNA levels in excess of 10-fold.[38] It was unclear, however, whether this was due to low fibrinogen levels or the fibrinogen breakdown products generated as a result of disseminated intravascular coagulation. A subsequent study, investigating the effect of purified plasmic fragments of fibrinogen on fibrinogen synthesis in the rat, suggested that the fibrinogen degradation products themselves induced increased fibrinogen synthesis.[60] It was shown that intravenous or intraperitoneal injection of purified fragment D (in concentrations equivalent to those seen with lysis of approximately 8% of intravascular fibrinogen) resulted in a four-fold increase in the synthesis of fibrinogen. Significantly, fragment D did not affect the level of other acute phase reactant proteins, and therefore appears to be specific for fibrinogen. This suggests that plasmin fragments of fibrinogen may well provide a feedback mechanism which may regulate fibrinogen synthesis.[60]

Fibrin formation

The fibrin clot promotes haemostasis by providing a structural support and scaffholding for cellular and other blood components which constitute the thrombus in vivo. In the final step of the coagulation cascade, soluble fibrinogen is cleaved by thrombin, resulting in the spontaneous polymerisation of the altered fibrinogen molecules to form the long threadlike polymer, fibrin. The process of fibrin formation is broadly divided into three steps (see figure 3) each of which will be considered separately below. These include:

(1) Thombin cleavage of fibrinogen
(2) Non-covalent assembly of fibrin monomers
(3) Covalent crosslinking of fibrin monomers catalysed by factor XIIIa.

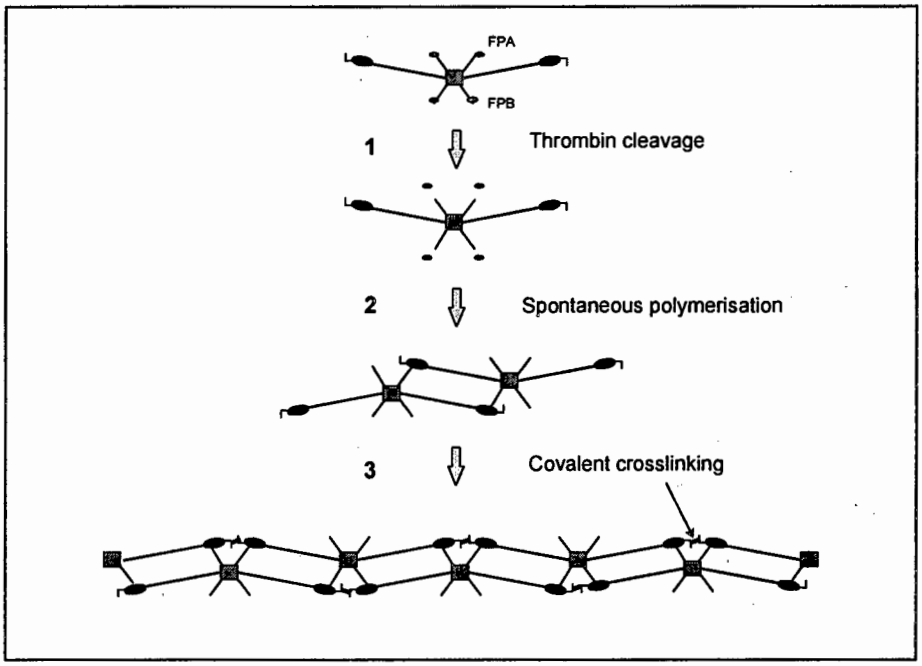


Figure 3. *A schematic representation of the three basic steps in the process of fibrin formation (1) Thombin cleaves fibrinogen releasing fibrinopeptides A and B. (2) This exposes polymerisation sites on fibrin monomer resulting in the spontaneous, non-covalent polymerisation of fibrin monomers (3) The fibrin polymer is stabilised by covalent crosslinking of γ chains catalysed by factor XIIIa.*

Thrombin cleavage of fibrinogen

Whilst thrombin is best known for its role in the conversion of fibrinogen to fibrin, it is in fact a multifunctional protease whose varied actions, both on cells and soluble protein substrates, may influence several important (patho)physiological processes (figure 4). Thrombin is a potent activator of platelet aggregation,[61,62] it is chemotactic for monocytes,[63] mitogenic for lymphocytes and mesenchymal cells (including vascular smooth muscle cells),[64-66] and has a number of important effects on the vascular endothelium; these include the stimulation of endothelial cell production of prostacyclin,[67] platelet activating factor (PAF),[68] plasminogen activator inhibitor (PAI),[69] and platelet derived growth factor (PDGF).[70] Thrombin also induces increased endothelial cell adhesiveness for neutrophils.[71]

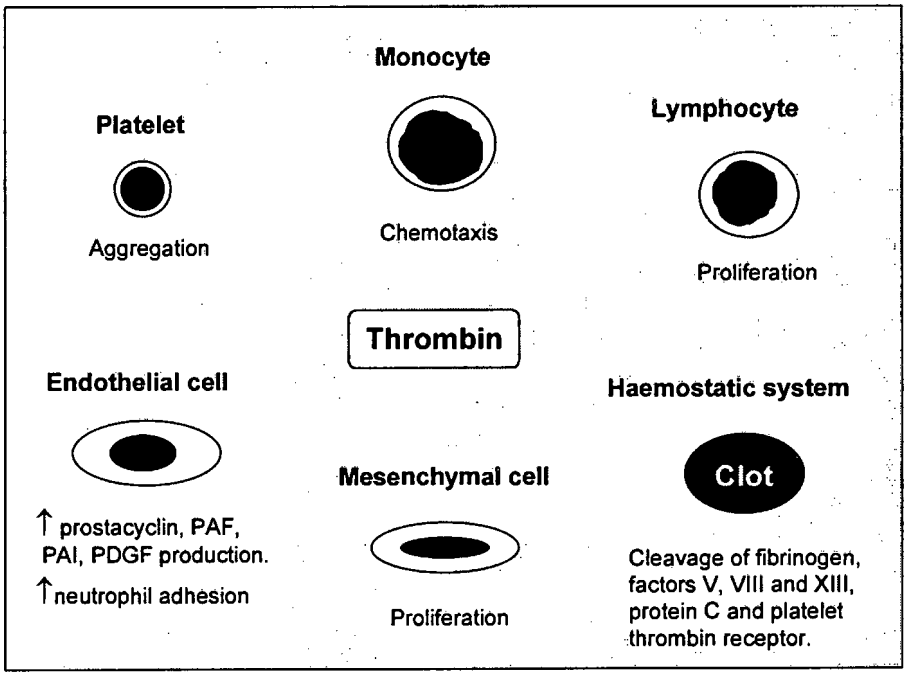


Figure 4. *An illustration of the varied actions of thrombin on cells and protein substrates which influence a number of important biological processes.*

It has numerous actions on proteins of the haemostatic system including the conversion of fibrinogen to fibrin monomers, as well as the proteolytic processing of factor V,[72] factor VIII,[73] factor XIII,[74] protein C,[75] and the platelet thrombin receptor.[76]

Thrombin interacts with fibrin(ogen) via two types of complementary binding sites on the fibrin(ogen) molecule. The first, the fibrinogen *substrate site* (figure 5a), interacts with the thrombin active site facilitating the cleavage of fibrinogen to form fibrin monomers.[77-81] The second type of site on fibrin is not associated with proteolysis and is commonly referred to as a *non-substrate site* (figure 5b).[82-86]

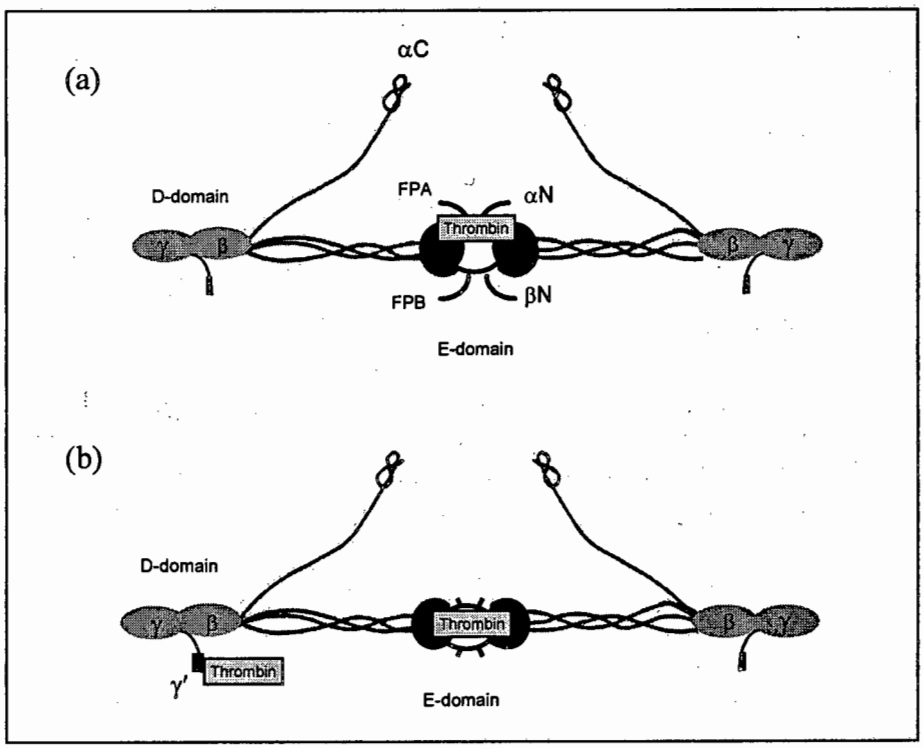


***Figure 5**. Thrombin interacts with fibrin(ogen) via two types of complementary binding sites on the fibrin(ogen) molecule: (a) The **substrate site**, which is located at amino terminal residues Aα 7-16, interacts with thrombin facilitating the cleavage fibrinopeptides A and B. (b) **Non-substrate sites** of high and low affinity are present on fibrin. The high affinity site (also present in fibrinogen) is situated in the D-domain of the γ'-chain. The low affinity sites are situated in the fibrin E domain.*

The thrombin active site has two separate domains: a *catalytic* site (which includes the Asp-His-Ser catalytic residues, the specificity pocket and an apolar pocket) and an extended *fibrinogen recognition site.*[87,88] Thrombin binding to the fibrinogen substrate site (amino terminal residues Aα 7-16) is mediated through the anion binding *fibrinogen recognition site* which is situated in an extended patch of positively charged residues in the region of thrombin's loop segment centred around Lys 70-Glu 80.[89,90] The side chain of Arg-16 is inserted into the specificity pocket of thrombin[91,92] and, following cleavage at an RG bond of the Aα (Aα16/17) and Bβ chains (Bβ14/15), fibrinopeptides A and B are released.[77-81] The release of FPA and B results in the formation of intermediate fibrin monomers which expose new binding sites.[93] Whilst the release of fibrinopeptide A occurs rapidly and is independent of fibrin polymerisation, cleavage of the Bβ chain is much slower and is largely dependent on fibrin polymerisation.[94-96]

Thrombin also associates with fibrin via *non-substrate sites*[82-86] (figure 5b) and this appears to play an important role in the regulation of fibrin assembly. Fibrin possesses two types of non-substrate thrombin binding sites, one of high affinity and the other of low affinity.[83,97] The high affinity site, which is present in fibrinogen as well as fibrin, is located exclusively in the D-domains of the γ′ variant* of the gamma chain, whilst the low affinity sites are situated in the fibrin E domain, and may represent a residual aspect of the substrate recognition sites in fibrinogen.[97] Thrombin binding to fibrin non-substrate sites appears to have several physiologically important consequences. It has been suggested that this binding, by limiting the diffusion of thrombin, plays an important role regulating clot propagation, as patients whose fibrin lacks one or more of the non-substrate binding sites have a tendency to develop recurrent thromboembolic disease.[98-100] In addition, fibrin bound thrombin is resistant to inhibition by circulating heparin-antithrombin III complexes.[101,102] This might explain the limited effectiveness of heparin in preventing coronary reocclusion after successful treatment with the thrombolytic, tissue plasminogen activator (t-PA).[103-106]

* The γ′ variant is a structural variant of the fibrinogen γ-chain and is present in about 10% of plasma fibrinogen molecules.

Thrombin binding to non-substrate sites on fibrin also appears to play an important role in accelerating fibrin assembly by a mechanism that is not dependent on thrombin's catalytic activity.[82] Finally, the fibrin-thrombin complex, formed as a result of thrombin binding at the fibrin non-substrate site, accelerates the activation of factor XIII, thereby upregulating the process of fibrin matrix assembly.[107]

Non-covalent polymerisation

Following the cleavage of the fibrinopeptides A and B, the fibrin monomers polymerise spontaneously to form a fibrin clot.[108] The newly exposed sites (figure 6) in the E domain link up with the D-domains on neighbouring molecules via non-covalent interactions.[78,79,81] The polymerisation process comprises a number of different interactions: Two sites on the E domain (E_A and E_B) are involved in interactions with two corresponding sites on the D-domain (Da and Db respectively). In addition, two other important types of association occur between fibrin monomers. One involves the association of D-domains (D:D associations) whilst the other involves association of the carboxyl termini of the Aα chains (the αC domains) of neighbouring molecules.

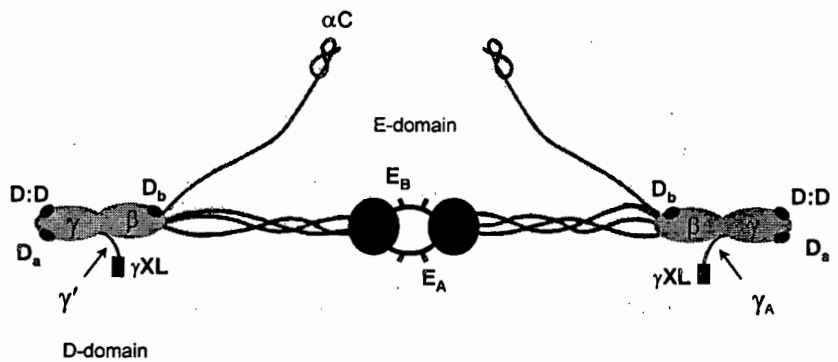


Figure 6. *Thrombin cleavage of fibrinogen exposes several polymerisation sites on the fibrin monomer. Two sites on the E domain (E_A and E_B) are involved in interactions with two corresponding sites on the D-domain (Da and Db respectively). Other polymerisation sites exposed include sites on the D-domain involved in D:D associations, as well as the carboxy-termini of the Aα chains (αC).*

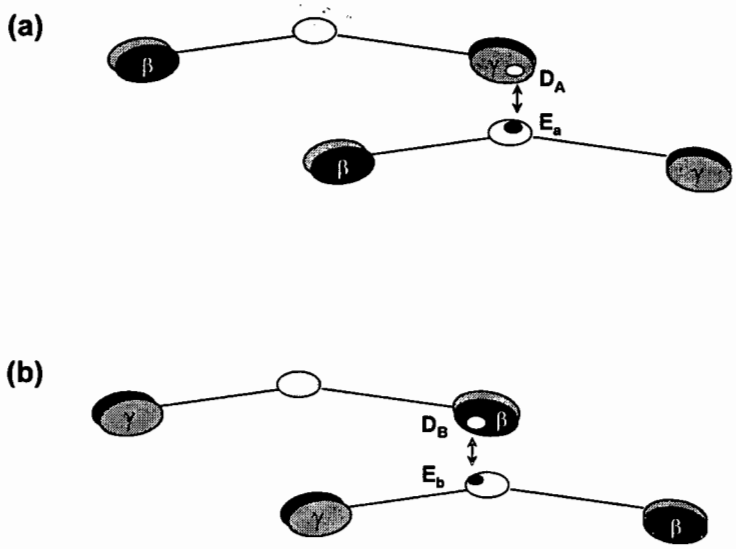
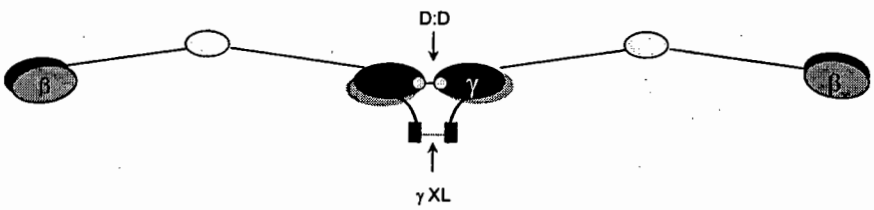


Figure 7. *A schematic representation of the interactions involved in fibrin polymerisation. (a) Cleavage of fibrinopeptide A exposes the primary polymerisation site, E_A, which interacts with a complementary site, Da, situated at the carboxyl terminal segment of the γ-chain in the D-domain. This interaction results in the formation of double stranded fibrils. (b) The cleavage of fibrinopeptide B exposes a second polymerisation site, E_B which interacts with a complementary site situated in a carboxyl terminal β-chain segment of the D-domain, known as the Db site.*

Fibrin assembly is initiated by the exposure of the *polymerisation site* E_A following the cleavage of the amino terminal region of the Aα chain.[78,79,81] This site is composed of at least two peptide sequences, one of which is located at the amino terminal end of the fibrin Aα chain, the Gly-Pro-Arg sequence (Aα17-20),[109,110] whilst the other is in the amino terminal region of the fibrinogen Bβ chain (Bβ15-42).[111] The newly exposed E_A site subsequently associates with a complementary binding site on the D-domain, Da,[112-115] which is situated in the carboxyl terminal γ chain segment (γ337-339)[116,117] (figure 7a). These Da:E_A associations result in the formation of double stranded fibrils, with fibrin molecules arranged in an 'end-to-middle' domain staggered overlapping fashion (see figure 3).[108,118-120] The cleavage of fibrinopeptide B, which occurs more slowly than that of fibrinopeptide A and is dependent on fibrin polymerisation,[78,79,81] exposes a second polymerisation site, E_B,[121] beginning with the Bβ15-18 sequence, GHRP.[109,110] This site interacts with a

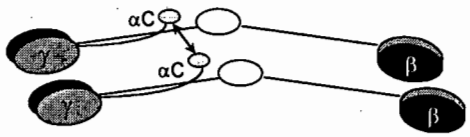
complementary site situated in a carboxyl terminal β-chain segment of the D-domain, known as the Db site (figure 7b).[114,121] Although the E_B:Db interaction contributes to the formation of lateral associations between fibrin strands, it is not an absolute requirement for this process.[121-122]

Associations between the D domains on neighbouring molecules involve two distinct and recently defined *self association sites* on the fibrinogen D domain. One overlaps with, and is part of, the γ chain crosslinking site in the middle portion of the D domain, and the other is situated at the outer end of each D domain (Figure 7c). [123-125] These sites associate with corresponding regions on adjacent fibrinogen molecules and these interactions appear to be essential for proper alignment of fibrin molecules in the assembling polymers as well as branching of fibres.[124]



(c) The D-domains on neighbouring molecules associate with one another via two self association sites, namely (i) the γ chain crosslinking site in the middle portion of the D domain and (ii) a site situated at the outer end of each D domain.

In addition, associations between the carboxyl termini of the Aα chains (the αC domains) also play an important role fibrin assembly.[126,127] Prior to proteolytic attack by thrombin, the αC domains tend to be associated non-covalently with the fibrinogen E domain; however, following thrombin cleavage of FPB, the αC domains become untethered and available for interaction with other αC domains (figure 7d), thereby promoting lateral fibril associations and fibrin assembly.[128,129]



(d) Interactions between the carboxyl-termini of the Aα chains (the αC domains) promote lateral fibril associations.

In summary, fibrin strands initially align spontaneously through D:E interactions which are supported by end-to-end D:D interactions. Subsequently and concommitantly fibrin strands undergo lateral associations via a combination of D:E, D:D and αC:αC interactions resulting in the thickening and branching of fibres giving rise to the meshwork of fibres that constitutes the fibrin clot (see figure 9).

Covalent crosslinking

Fibrin clots held together by non-covalent interactions alone are easily disrupted when subjected to the forces of stress and strain associated with blood flow through the vasculature.[130] However, the incorporation of covalent bonds between the fibrin units in the assembled matrix, dramatically alters the mechanical properties of the clot, providing structural stability, integrity and elasticity to an otherwise easily deformable fibrin clot.[131,132] Factor XIIIa, catalyses the covalent stabilisation of fibrin, via formation of peptide linkages between the ε-NH_2 group of certain *lysine* residues and the γ-$CONH_2$ of certain *glutamines*, with the consequent formation of ε-(γ-glu)lys isopeptide bonds.[133-134] Each γ-chain contains both a glutamine (residue 398 or 399) and a lysine (residue 406) which undergo reciprocal crosslinking with another γ-chain (figure 8), orientating

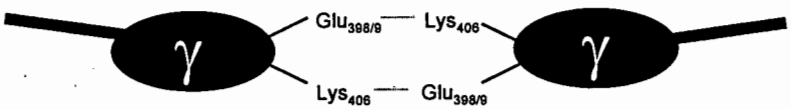


Figure 8. *The factor XIII catalysed covalent stabilisation of fibrin via reciprocal peptide linkages between γ chain glutamine (398 or 399) and lysine (406) residues.*

the two polypeptides in a symmetrical anti-parallel manner to form a γ-dimer.[135-137] Whilst γ-chain crosslinking has long been thought to involve end-to-end crosslinking of adjacent D-domains, recent studies suggest that transverse crosslinking between γ-chains on *adjacent fibrils* predominates.[138] Higher order forms of cross-linked γ chains, including γ trimers and γ tetramers also occur providing stability to branched structures.[139,140]

In addition to γ chain crosslinking, crosslinking among α chains (which occurs more slowly) generates oligomers and larger α chain polymers which also play an important role in the stabilisation of fibrin clots.[141] Factor XIIIa also catalyses the crosslinking of α chains to γ chains.[139,140] A variety of other proteins may become crosslinked to fibrin. The most important include fibronectin, which may alter the mechanical properties of the clot,[142] and the plasmin inhibitor, α_2-antiplasmin (α_2PI), whose efficiency increases when it is crosslinked to fibrin.[143] Another property conferred by factor XIIIa crosslinking is a relative increase in the resistance of the fibrin gel to lysis by plasmin.

The final result of the fibrin assembly process is the generation of a three dimensional, highly interconnected meshwork of fibrin fibres which function as a haemostatic plug (figure 9).

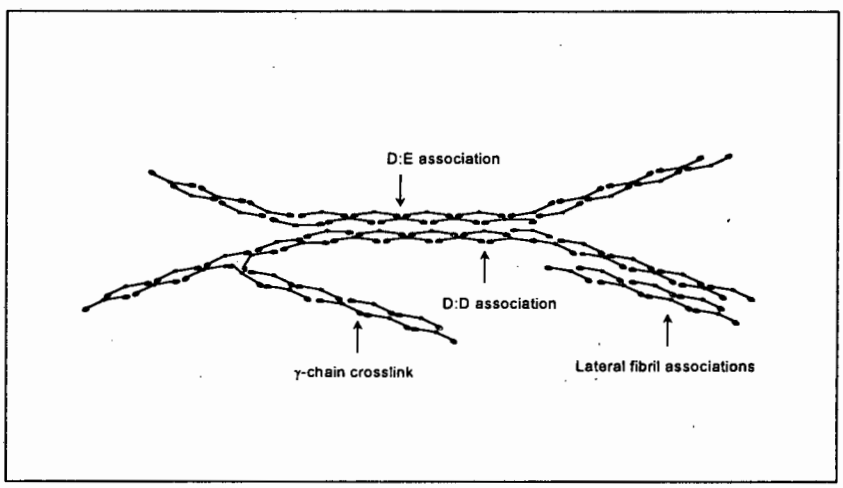


Figure 9. *A schematic representation of the branched structure of fibrin which results from D:D, D:E, αC:αC and γ- and α-chain crosslinking reactions.*

Plasmin mediated fibrin(ogen)olysis

The fibrin clot is not a permanent structure and once it has fulfilled its role in achieving haemostasis it must be dismantled. Thus, immediately following fibrin formation, processes are set in motion which are directed at its dissolution. These processes are as important as the formation of the clot itself, as failure of this system can lead to life-threatening thromboembolic events including myocardial infarction, cerebrovascular occlusion and pulmonary embolism. Fibrin dissolution is effected by the plasminolytic system which, under the control of a variety of activators and inhibitors, gives rise to the fibrinolytic enzyme plasmin.

Plasminogen activators

Plasminogen activators convert the circulating pro-enzyme plasminogen into the active fibrinolytic protease, plasmin. Two major plasminogen activators have been identified in mammals: tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA).[144] Both t-PA (70 kDa) and uPA (55 kDa) are serine proteases, encoded by separate genes. The two plasminogen activators possess distinct targeting determinants in their non-catalytic regions: the "growth factor domain" of u-PA facilitates the binding of u-PA to specific receptors on the plasma membrane of a variety of cell types,[145,146] whilst specific structural domains in t-PA enhance its binding to fibrin and other components of the extracellular matrix.[147] Cell surface expression of u-PA, through the localised generation of plasmin, is thought to play an important role in a number of physiological as well as pathological processes, including cell migration, tissue remodelling and metastasis.[148] On the other hand, t-PA appears to be the major plasminogen activator at sites of fibrin formation due to its high affinity for fibrin.[149] The adsorption of tPA together with plasminogen on the fibrin fibre surface allows the neccessary contact required to effect optimal activation rates for plasmin production;[150] fibrin binding confers upon tPA an approximate 1000-fold increase in its affinity for plasminogen.[151] Two sites in fibrin are involved in the binding and

upregulation of t-PA, namely Aα148-160 and γ312-324.[152-155] These sites, which are inaccessible in fibrinogen, become exposed following the conversion of fibrinogen to fibrin.[156,157]
A group of inhibitors have evolved to keep the plasminogen activators in check. These include plasminogen activator inhibitors 1 and 2 (PAI 1 and 2) and protease-nexin 1.[158] PAI-1 is the major plasminogen activator inhibitor in the plasma, having a high affinity for both tPA and uPA, whilst the latter two inhibitors are predominantly directed at uPA.[159]

Plasminogen and plasmin

Plasmin is a relatively broad spectrum serine protease and, whilst its primary substrate is fibrin, it is also capable of degrading most extracellular matrix (ECM) proteins.[160] Thus, in addition to its role in fibrinolysis, plasmin may play an important additional role in ECM degradation. Its inactive precursor, plasminogen, is a glycoprotein of approximately 92-94 kDa[161] and is present in the plasma and extracellular fluids at a concentration of 1-2 μM.[159] Plasminogen consists of a heavy amino terminal *non-catalytic* region made up of five homologous domains of triple looped, disulphide bridged structures commonly known as kringles,[162] and a lighter *catalytic* C-terminal domain (Figure10).[161] The kringle regions contain the lysine binding sites which are involved in binding of plasminogen and plasmin to fibrin and a number of other macromolecules.[163,164]
Plasminogen, in its native form, has one high affinity lysine binding site (on the first kringle domain), its major binding site for fibrin, and approximately 5 sites of lower affinity.[165-167] Upon binding to lysine, plasminogen undergoes a conformational change from a closed, compact structure, in which the 5 kringle domains and 2 domains from the serine protease region are in close contact with each other, to an open extended structure with these domains no longer closely associated.[168] This conformational change renders plasminogen far more susceptible to proteolytic activation by plasminogen activators[169] and, following their intimate contact on the fibrin surface, plasminogen is cleaved at an Arg-Val bond, to yield the active, disulphide linked two chain serine protease, plasmin.[170]
Alpha-2-antiplasmin serves as the primary inhibitor of plasmin in the

plasma.[171] However, when plasmin is generated on the clot surface it is relatively protected from inactivation by this inhibitor.[172] This helps to localise plasmin activity to the immediate vicinity of the fibrin clot, allowing t-PA to initiate clot lysis without inducing a systemic fibrinolytic state.

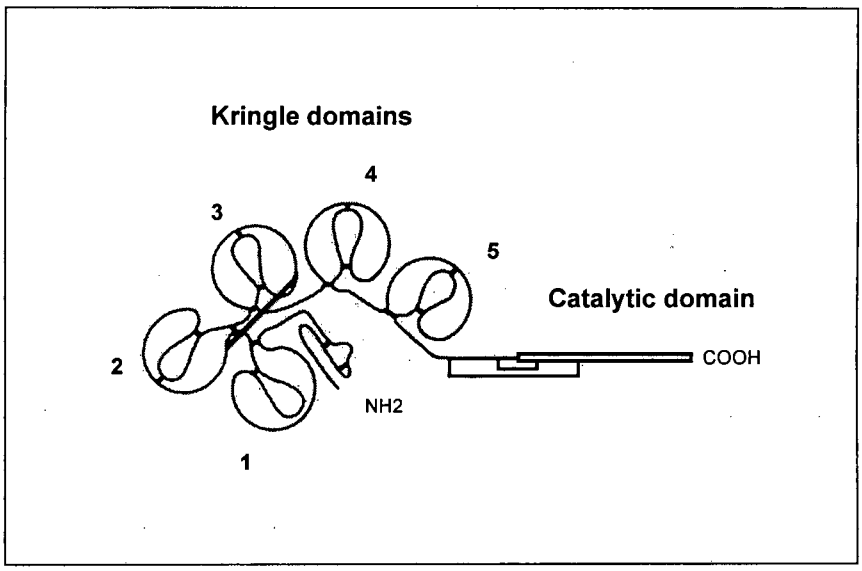


Figure 10. *A schematic representation of the plasminogen molecule illustrating the heavy NH_2-terminal non-catalytic domain comprising kringle loops and a lighter catalytic COOH-terminal domain.*

Fibrin(ogen) degradation

The architecture of the fibrin clot is as suited to its dissolution, as the structure of fibrinogen is suited to polymerisation. This is due to the accessibility and vulnerability of essential connections between the nodular bodies (D and E domains) to proteolysis. As intermolecular contacts between fibrin monomers are limited to the nodular bodies, channels to the interdomainal connectors are left open, facilitating the diffusion of proteolytic agents throughout the fibrin clot. Moreover, rather than having to cut all the way through a solid polymer, plasmin need only cleave the slender three strand

connectors. Consequently, the dissolution of a fibrin clot requires lysis of a relatively small number of bonds. Intermediate polymers are formed by the cleavage of any two connectors along the length of a fibril. Proteolytic digestion then continues until these intermediate polymers are reduced to core fragments.[173]

As plasmin degradation of fibrinogen yields products that are very similar to those that it generates from fibrin,[174,175] it is helpful to consider plasmin degradation of fibrinogen prior to that of fibrin. The major differences between the products of fibrinogen and fibrin digests reflect the covalent crosslinking associated with factor XIIIa stabilised fibrin.[176,177]

Prolonged exposure of fibrinogen to plasmin can ultimately catalyse the cleavage of nearly 60 bonds, although only 5 to 6 of these bonds are cleaved rapidly.[178] Degradation of fibrinogen begins in the Aα chain (at several sites in the C-terminal region) and is shortly followed by degradation of the Bβ chain (where the Bβ1-42 peptide is cleaved from the N-terminus).[179] The γ chain is the most resistant to degradation and the last to degrade.[180]

Degradation initially results in the release of the Aα peptides, fragments A, B and C (with molecular weights of approximately 15kDa), leaving the residual intermediate fibrinogen product, fragment X (240-265 kDa), which may exist in a number of forms depending on the extent to which the Aα and Bβ chains are degraded (Figure 11).[180] As degradation continues, the size of fragment X becomes progressively smaller, giving rise to discreet fragments called D (83-100 kDa), E (41 kDa) and a transient fragment Y (approximately 150 kDa) which consists of a D-E core. Further degradation of Y yields the terminal fragments D and E.[180]
Fragment D contains extensively degraded Aα chains, partially degraded Bβ chains, and γ chains at various stages of degradation. Depending on the degree of γ chain degradation, fragments D are designated D_1, D_2 or D_3 (with D_1 having the least, and D_3 the most, extensively degraded γ chains).[175,180,181] However, as extracellular γ chain digestion by plasmin does not occur in the presence of calcium ions, it is likely that fragment D_1 is the only physiologically important

D-species in vivo.[182] Fragment E contains extensively degraded Aα, Bβ and γ chains which are linked through disulphide bonds.[180]
The terminal degradation products generated by plasmic degradation of *fibrin* show slight differences to those described above, largely as a result of crosslinking of fibrin monomers by factor XIIIa. Covalent crosslinking of antiparallel γ chains results in the formation of the dimeric degradation product, DD (known as D-dimer), rather than the

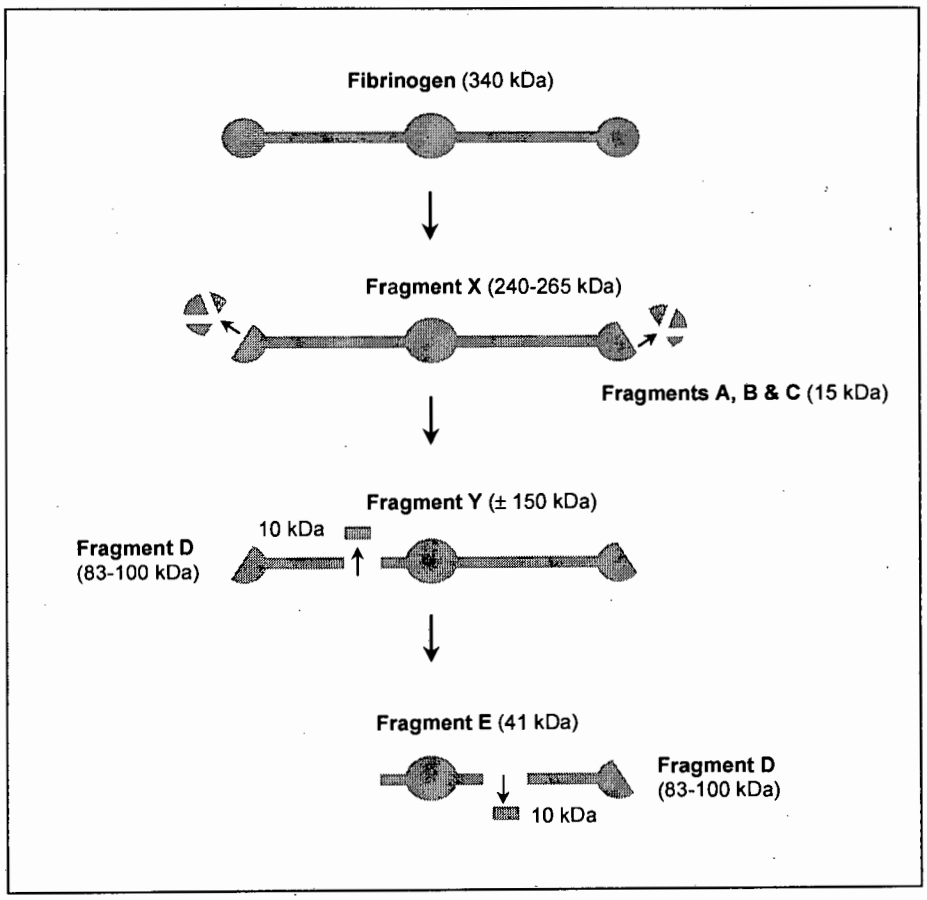


Figure 11. *A schematic representation of plasmin mediated fibrinogenolysis. Degradation of fibrinogen initially releases the Aα peptides, fragments A, B and C. The residual intermediate fibrinogen product, fragment X may exist in a number of forms depending on the extent of Aα- and Bβ-chain degradation. With continued degradation, fragment X gives rise to fragments D (83-100 kDa), E (41 kDa) and a transient fragment Y (± 150kDa) which consists of a D-E core. Further degradation of Y yields the terminal fragments D and E.*

individual D fragments,[176] and the non-covalent interaction of the central E domain with two distal D domains is sufficiently strong for the generation of the heterotrimer D_2E (figure 12).[183] In addition, monomeric fragment E has been identified as a major soluble derivative of crosslinked fibrin, although this does differ slightly from fragment E derived from fibrinogen.[184,185]

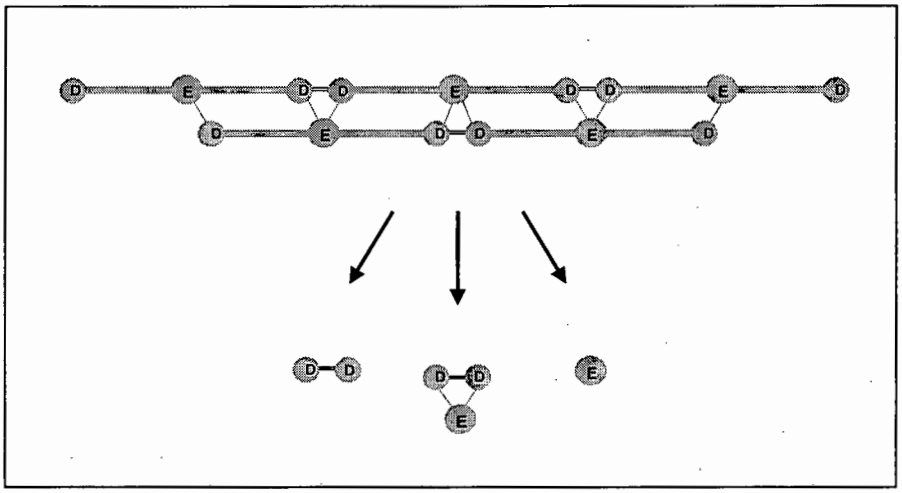


Figure 12. *The terminal degradation products generated by plasmic degradation of fibrin. As a result of covalent crosslinking of γ-chains as well as non-covalent interactions between E and D domains, plasmic digestion of fibrin yields terminal degradation products which differ from those generated from fibrinogen. These products include D-dimer (DD), the heterotrimer D_2E and soluble monomeric fragment E.*

Although these fragments appear to be the major soluble products arising from the digestion of crosslinked fibrin, *in vitro*, larger soluble intermediate degradation products are more likely to circulate during fibrinolysis in vivo.[186] Fibrinolysis in vivo appears to be a stepwise process involving the following sequence of events. Initially, polypeptide chain cleavages occur which do not disrupt the solid clot matrix. Further degradation generates high molecular weight derivatives which remain associated with the clot. Continued degradation then results in the release of several of these non-covalently bound derivatives into solution. Further degradation of these products in solution eventually yields the terminal products DD, D_2E and E.[186] This appears to occur in progressive cycles of

proteolysis of accessible superficial regions of the clot, allowing for layer by layer dissolution of thrombi without the release of partially lysed, but insoluble material into the circulation.[186-188] This ensures that partially lysed, but insoluble material does not enter the circulation, thereby reducing the risk of thromboembolic events following fibrinolysis.

Non plasmin mediated fibrin(ogen)olysis

Although plasmin mediated fibrinolysis constitutes the primary pathway for fibrin dissolution, several alternative pathways have been identified. These are largely cell mediated and, in particular, involve neutrophils[189-197] and monocyte/macrophages.[198-199]

The ability of leukocytes to degrade fibrin clots was first noted at the turn of the century by Rulot and Opie who found that fibrin dissolution was greatly enhanced in the presence of leukocytes.[200-201] Since then the intimate association of leukocytes with fibrin deposits has frequently been noted both intra- and extravascularly. Within the *vascular compartment*, leukocytes frequently accumulate within complex thrombi consisting of fibrin and platelets.[202-204] At *extravascular sites*, the migration of neutrophils to sites of injury where fibrin has accumulated is a central feature of the inflammatory response.[204-206] Fibrinopeptide B, released during fibrin formation, has been shown to enhance neutrophil chemotaxis and facilitates neutrophil migration towards the fibrin clot.[207] Once localised within fibrin deposits, the direct interaction of neutrophils with fibrin molecules may induce the release of granule proteases which can then digest fibrin.[190]

The neutrophil proteases responsible for fibrin(ogen) degradation were distinguished from plasmin on the basis of several observations. Firstly, neutrophils were shown to contain only very low levels of plasminogen, insufficient to effect fibrinolysis.[95,208,209] Secondly, fibrinogen degradation products generated by leukocyte extracts were shown to be structurally and immunochemically distinct from those produced by plasmin.[191,195,210] Finally, plasmin(ogen) inhibitors and

activators did not influence the fibrinolytic activity of leukocyte extracts.[195,211] It was subsequently revealed that the two major proteases in leukocyte extracts, responsible for fibrin(ogen) degradation, were human neutrophil elastase (HNE) and cathepsin G.[212-214] As these proteases reside primarily in the neutrophil granules, potential mechanisms for the activation of the neutrophil fibrinolytic pathway include neutrophil degranulation, phagocytosis, cell lysis as well as fibrin formation itself. Recent studies have shown that these granule proteases can further process the terminal products of plasmic fibrin degradation.[215,216] HNE degradation of fibrinogen generates high molecular weight degradation products that are non-clottable, and several of these have been shown to be capable of competitively inhibiting thrombin cleavage of fibrinogen.[197] A characteristic feature of fibrinogen proteolysis by HNE is the release of the Aα1-21 peptide from the N-terminal region of the Aα chain of fibrinogen, and plasma levels of this peptide have been used as a marker of HNE activity *in vivo*.[217]

More recently, the physiological relevance of HNE mediated fibrin(ogen)olysis has been questioned, as relatively high concentrations of HNE are required for significant fibrinolysis in vivo.[193] Moreover, the presence of high affinity inhibitors of HNE in the extracellular space,[218] has cast further doubt over the direct role of HNE in neutrophil mediated fibrinolysis. This has turned attention to the indirect roles of HNE in fibrinolysis, in particular its modulatory effects on the plasminogen activator/plasmin system, a number of which require significantly lower concentrations of HNE.[193,219] For instance, HNE used at relatively low concentrations has been reported to cleave, and thereby inactivate, two major inhibitors of the plasminolytic system, namely the plasmin inhibitor, alpha-2-antiplasmin,[219] and the plasminogen activator inhibitor, PAI-1.[193] In addition, HNE has been shown to cleave plasminogen to miniplasminogen, which is more susceptible to cleavage by plasminogen activators,[220] and less susceptible to inhibition by alpha-2-antiplasmin.[221] Cathepsin G may also play an indirect role in the regulation of the fibrinolytic pathway through the cleavage and activation of u-PA.[222]

Despite controversy over the direct roles of HNE and cathepsin G in neutrophil mediated fibrinolysis, considerable evidence has now accumulated to suggest that these proteases may, in fact, be able to escape inhibition by naturally occurring protease inhibitors and focus their activity in the immediate pericellular environment and at sites of fibrin formation. Firstly, HNE, once bound to a fibrin clot has been found to escape inhibition by naturally occurring proteinase inhibitors.[223] Secondly, neutrophil adhesion to fibrin, via integrin receptors, has been found to create zones of close contact between the neutrophil and fibrin (so-called "protected compartments") which prevent access of plasma anti-proteinases to HNE released at the cell-substrate interface.[217,224] Finally, it has been shown that, following degranulation, HNE and cathepsin G bind to the neutrophil membrane where they remain catalytically active but resistant to naturally occurring proteinase inhibitors.[225-229] In this way the neutrophil is able to focus and preserve HNE activity on the cell surface allowing HNE to play a direct role in fibrinolysis.

In addition to its pro-fibrinolytic activity, HNE has recently been reported to inhibit plasmin-mediated fibrinolysis. This is due to its proteolytic action on fibrin which results in a diminution of the stimulatory effect of fibrin on t-PA mediated plasminogen activation.[194,230] It has been suggested that this effect may, in fact, dominate the pro-fibrinolytic effects of HNE. Precisely which is the dominant role for HNE in fibrinolysis *in vivo* remains unclear, as data obtained *in vitro* has tended to vary with experimental design. For instance, whereas many of the earlier experiments investigated the effect of HNE on fibrinogen or fibrin clots used in isolation, the latter study tested the effect of HNE on plasmin-mediated lysis of whole blood clots.

In addition to their role in fibrin(ogen)olysis HNE and cathepsin G can also influence the coagulation pathway by the proteolytic modification of a variety of other clotting factors including factors V, VII, IX, X and protein S.[231-234]

Recently, a 600 kDa neutrophil membrane associated protease was reported to degrade fibrinogen.[235] This protease was shown to render fibrinogen non-clottable, a process considered to be important in limiting fibrin deposition at sites of inflammation. It was later shown

to degrade fibrin clots,[236] leading to the suggestion that neutrophil mediated fibrinolysis is not necessarily dependent on neutrophil degranulation.[236] As with HNE, this protease was reported to cleave the Aα 1-21 peptide from the N-terminus of the Aα chain of fibrinogen.[235] Both fibrinogen and fibrin degradation products generated by this protease appear to have biological activity. Low molecular weight *fibrinogen* peptides generated by this protease have been reported to associate with neutrophil β_2–integrin receptors modulating neutrophil adhesion to immobilised fibrinogen.[237] *Fibrin* degradation products generated by this protease also possess biological activity. Low molecular weight peptides inhibit thrombin induced platelet aggregation whilst higher molecular weight products partially overcome platelet induced resistance of fibrin to plasmic lysis.[238]

In addition to the contribution made by granule and membrane associated proteases, neutrophils may also enhance fibrinolysis by expression of plasminogen activators on their surface, in particular u-PA.[239]

The monocyte/macrophage is also thought to play an important role in fibrinolysis. It is capable of assembling the components of the plasminogen activator system which ultimately results in the generation of plasmin.[240,241] In addition, macrophage degradation of fibrin may also occur via a plasmin independent pathway. This alternative pathway involves the binding of fibrinogen to the cell surface integrin CD11b/CD18 (Mac1), followed by internalisation and trafficking to the lysosome where the aspartyl protease, cathepsin D, degrades the protein.[198] Another potential mechanism of non-plasmin mediated fibrinolysis is the uptake of fibrin monomer into macrophage lysosomes through a process involving the interaction of the amino-terminus of the alpha chain of fibrin with a specific cell surface receptor.[242]

Thus there appears to exist a number of leukocyte mediated mechanisms which may provide alternatives to the plasmin-mediated fibrinolytic system. The notion of physiologically relevant non-plasmin mediated fibrinolytic pathway(s) has gained support from two recent sets of studies performed on animal and man. The first

reported that patients with a total congenital deficiency of plasminogen survived without any major thromboembolism but suffered from numerous inflammatory disorders.[241] The second showed that endogenous lysis of pulmonary emboli does still occur (albeit relatively slowly) in animals devoid of t-PA and u-PA.[243,244]

Fibrinogen interactions

Fibrinogen is a multifunctional protein which, in addition to its role in haemostasis, can influence a variety of other physiological processes. Fibrin(ogen) has been shown to interact with a variety of cells including platelets, neutrophils, monocyte/macrophages, endothelial cells, smooth muscle cells and fibroblasts, often modulating important cellular events (figure 13). Fibrinogen also interacts with a number of extracellular matrix proteins including fibronectin whose incorporation into fibrin changes the physicochemical properties of the fibrin clot. In addition, fibrinogen binds to a variety of micro-organisms, including several pathogens, and may contribute to the ability of these organisms to invade host-tissues and evade host defences. Finally, through its ability to interact with various components of the coagulation and fibrinolytic systems (including thrombin, plasminogen, t-PA, and factor XIII), fibrin(ogen) is central to many of the important feedback mechanisms that regulate haemostasis.

Interaction with neutrophils

Neutrophil interaction with fibrin(ogen) has been the subject of intense interest over the last decade as events following this interaction have the potential to modulate both the inflammatory and coagulation responses. Fibrinogen binds specifically to integrin receptors on the neutrophil surface, predominantly via the β_2-integrins, CD11b/CD18 (Mac1)[245-247] and CD11c/CD18 (p150/95),[248] and to a lesser extent via the β_3-integrin, leukocyte response integrin.[249,250] Specific sequences on the fibrinogen molecule are recognised by these receptors, and the nature of the

stimulus appears to determine which integrin receptor is involved. For instance phorbol ester stimulated neutrophils adhere to surface adherent fibrinogen via CD11b/CD18 which recognises the K-Q-A-G-D-V sequence situated at the carboxyl terminus of the fibrinogen γ-chain.[245] In neutrophils stimulated with the chemotactic peptide fMLP, CD11b/CD18 recognises the γ-chain sequence Gly_{190}-Val_{202}.[246] In neutrophils stimulated with TNFα, CD11c/CD18 mediates binding to fibrinogen coated surfaces and recognises the G-P-R sequence at positions 17-19 of the NH_2 terminus of the α-chain.[248] The binding of neutrophils to surface adherent fibrinogen via β_2-integrin receptors induces tyrosine phosphorylation of certain neutrophil proteins[251] which, in turn, signal for the initiation of various important cellular events such as cell spreading,[252,253] the respiratory burst,[254] and degranulation.[255]

Neutrophils stimulated with FMLP have been reported to recognise the γ-chain sequence γ190-202 on both immobilised and soluble fibrinogen.[246] Recent studies suggest that this region, together with the γ-chain sequence γ377-395, form a binding pocket for the I-domain of CD11b/CD18.[256] The β_3-integrin receptor, leukocyte response integrin (LRI), recognises soluble fibrinogen both via the R-G-D sequence of the fibrinogen α-chain as well as the K-Q-A-G-D-V sequence of the carboxyl terminus of the fibrinogen γ-chain.[249,250] Soluble fibrinogen has been reported to inhibit neutrophil chemotactic activity and oxygen consumption in response to certain stimuli.[257] Several fibrin(ogen) degradation products (FDP) have also been found to modulate neutrophil function. Fibrinopeptide B has been shown to enhance neutrophil chemotaxis,[207] whilst plasmin generated fibrinogen degradation products have been reported to inhibit neutrophil chemotaxis, respiratory burst, bactericidal activity,[258] and adhesion to EC and protein coated surfaces.[259]

Fibrin degradation products are also capable of modulating neutrophil function. Plasmin derived D-dimer and D-monomer have recently been shown to be potent neutrophil chemoattractants.[260] HNE degradation of fibrin also generates products which are chemotactic for neutrophils.[261] Moreover, secondary digestion by HNE of plasmin derived FDP produces molecules that are more potent chemoattractants than native plasmin derived FDP.[261] The

fibrin matrix itself has been shown to inhibit neutrophil chemotaxis in neutrophils stimulated with FMLP or TNFα,[262] and this appears to be due to strong interactions between fibrin and the neutrophil β_1-integrin receptors, $\alpha_5\beta_1$.[263]

Interaction of fibrinogen and fibrin with the neutrophil results in the release of the recently reported 600 kDa membrane associated protease into the surrounding medium.[235-238] It has been suggested that this interaction occurs near the CD11c/CD18 receptor as monoclonal antibodies directed against this receptor cause marked inhibition of fibrin(ogen)olysis.[237,238] The fibrin(ogen) degradation products generated by the membrane associated protease have been shown to modulate both neutrophil and platelet function (see page 24).[237,238]

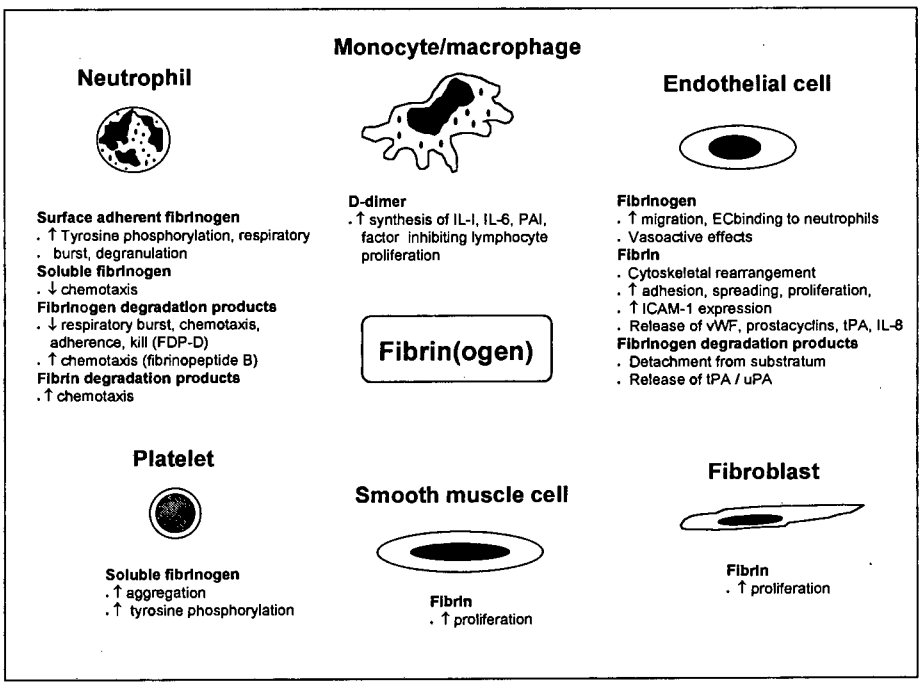


Figure 13. *A schematic representation of the various ways in which fibrin(ogen) and its products may influence cellular processes.*

Interaction with monocyte/macrophages

The contribution of macrophages to the clearance of microparticulate fibrin from the bloodstream has long been recognised. Disseminated intravascular coagulation, induced in various animal experimental models, has been shown to be associated with the rapid accumulation of particulate fibrin within the macrophages of the liver (Kuppfer cells), spleen and lung.[264,265] In vivo studies using guinea pig macrophages have demonstrated binding of fibrinogen and fibrin to these cells.[266] Later studies showed macrophages to bind fibrin with a much higher affinity than fibrinogen via a process that appeared to be receptor mediated.[267] Further studies have demonstrated absorptive endocytosis of fibrin monomer (but not fibrinogen) by rabbit peritoneal macrophages via a cell surface receptor which interacts specifically with the Aα N-terminal sequence G-P-R, exposed following the cleavage of fibrinopeptide A.[241]

More recently, fibrinogen has been reported to associate specifically, and calcium dependently, with activated monocytes.[268] This process is mediated by the integrin receptor CD11b/CD18 and internalisation of receptor bound fibrinogen leads to its degradation.[198]

The fibrin degradation product D-dimer has been reported to bind to CD11b/CD18 on the surface of monocytes, inducing the release of a factor which suppresses lymphocyte proliferation.[269] Following internalisation and degradation of DD, monocyte synthesis of biologically active IL-1β, IL-6, and PAIs is enhanced.[270]

Interaction with platelets

Platelet aggregation, with the subsequent formation of a platelet plug, is central to the process of haemostasis. The role of fibrinogen as a vital co-factor in platelet aggregation has long been known. Aggregation is mediated by the bridging of two integrin receptor molecules, $\alpha_{IIb}\beta_3$ (GPIIb/IIIa), by dimeric fibrinogen.[271] The regions of fibrinogen which bind to the $\alpha_{IIb}\beta_3$ receptor include the RGD sequence of the α-chain[272] as well as the dodecapeptide sequence 400-411 on the extreme carboxyterminus of the γ-chain.[273]

The platelet α-granule provides an important source of fibrinogen to be used during platelet aggregation, comprising 3-10% of total

platelet protein.[274] Recent data indicate that platelet fibrinogen is not synthesised by megakaryocytes (the nucleated precursors of platelets) as was previously thought, but is aquired exclusively from the plasma. This is suggested by the absence of α-granule fibrinogen in patients who lack the $\alpha_{IIb}\beta_3$ receptor,[275] and the ability of specific $\alpha_{IIb}\beta_3$ antagonists to inhibit fibrinogen uptake into alpha-granules.[276,277] Recent studies have reported that internalisation of surface bound fibrinogen by activated platelets may result in downregulation of platelet aggregation, suggesting an additional modulatory role for fibrinogen in haemostasis.[278] Finally, it has also been suggested that fibrinogen may modulate platelet function by inducing tyrosine phosphorylation of platelet proteins which is independent of platelet aggregation.[279]

Interaction with endothelial cells

Fibrinogen, fibrin and their respective degradation products have all been shown to interact with endothelial cells (EC), and are capable of modulating their activity. As fibrin deposition on vascular EC is a consistent feature of a variety of pathological processes (including trauma, infection, inflammation and neoplasia),[280] fibrin related products may influence EC behaviour in a number of clinical settings. Fibrinogen has been reported to bind via the RGD sequence of the α-chain to an EC protein immunologically related to the $\alpha_{IIb}\beta_3$ receptor on platelets, with the subsequent upregulation of EC migratory activity.[281] More recently, fibrinogen has been shown to bind to the EC adhesion molecule, ICAM1, which then serves as a bridging molecule enhancing leukocyte adhesion to the endothelium.[282] This raises the possibility that fibrinogen may play an important role in leukocyte-EC interactions in vivo. Fibrinogen also appears to have vasoactive effects and has been reported to cause ICAM1 dependent relaxation of saphenous vein rings at concentrations up to 2μM, with this relaxation being reversed at higher concentrations.[283]

Fibrinogen degradation product fragment D has been reported to induce the detachment of EC from the substratum, as well as inducing the release of t-PA and u-PA.[284] It was shown that the secretion of these plasminogen activators, via the generation of

plasmin, contributed to the proteolysis of the extracellular matrix and thus the EC detachment.[284]

Fibrin is also capable of binding specifically to vascular endothelium and has been reported to exert numerous effects. For instance, it has been reported to induce cytoskeletal reorganisation which is associated with a reversible disruption of EC monolayers,[285] as well as increased EC adhesion and spreading.[286] Fibrin also induces the release of several biologically active factors including von Willebrand factor,[287] prostacyclin, t-PA[288] and IL- 8.[289] Fibrin deposition can also induce new capillary formation *in vitro*[290] and an angiogenic response *in vivo*.[291] The exposure of the Bβ 15-42 sequence following thrombin cleavage of fibrinopeptide B, has been reported to enhance the spreading and proliferation of EC growing on fibrin matrices.[292] Recently, it has been shown that fibrin induces the upregulation of ICAM1 expression on EC, raising the possibility that the adhesiveness of ECs for leukocytes may be modulated at sites of fibrin deposition.[280]

Interaction with other cells

Fibrin(ogen) may contribute to the process of wound healing via its potent mitogenic effect on fibroblasts. This appears to be mediated by the binding of the Bβ-chain of fibrinogen to calcireticulin on the fibroblast surface.[293] Fibrin has also been shown to stimulate the proliferation of smooth muscle cells, whereas FDP, produced by the fibrinolytic action of these cells, appears to cause inhibition of proliferation.[294] As the proliferation of smooth muscle cells is central to pathogenesis of atherosclerosis,[295] fibrin may thus promote the generation of atherosclerotic lesions. Indeed, the presence of fibrin(ogen) in atherosclerotic plaques has been documented by several morphologic and immunohistochemical studies.[296-300]

Interaction with micro-organisms

Fibrinogen binding capacity is not unique to mammalian cells. Fibrinogen is also bound by a wide variety of micro-organisms and such binding may influence their pathogenicity.[301-314] Coating of the

cell surface with fibrinogen confers anti-phagocytic protective properties to certain micro-organisms. For instance, the binding of fibrinogen to the M protein of *group A streptococci* enhances its resistance to phagocytosis by neutrophils.[301,302] This effect has also been observed in animal hosts, where fibrinogen binding protects *streptococcus equinus subspecies equi.* from phagocytosis by equine neutrophils.[303] However, fibrinogen binding may also achieve the opposite effect, as observed in the case of *actinomyces pyogenes*, where fibrinogen coating enhances its uptake by bovine neutrophils.[304]

Binding to fibrin(ogen) in the extracellular matrix may facilitate the invasion of host tissues by certain micro-organisms. The interaction of *aspergillus fumigatus* with fibrinogen is thought to mediate attachment of the fungus to host tissues, thereby facilitating invasion.[305] A similar mechanism is proposed for *bacteroides gingivalis*,[306] and may also be used by *staphylococcus aureus*[307] and *candida albicans.*[308] Interestingly, the fibrinogen binding capacity of various strains of E. coli has been found to correlate with the pathogenicity of the organism.[309] It was suggested that this may be due to the facilitation of binding to ulcerated tissue or by preventing complement induced lysis of bacteria. Fibrinogen interactions with bacteria may also affect platelet function. Recently it has been shown that staphylococcus aureus induces platelet aggregation via a fibrinogen dependent mechanism which is independent of the $\alpha_{IIb}\beta_3$ fibrinogen binding domains.[311] It was suggested that this may play an important role in the pathogenesis of infective endocarditis, through the generation of infected thrombi on heart valves.

Interaction with calcium

The importance of calcium in fibrinogen polymerisation has long been known. Calcium accelerates fibrin clot formation and enhances the rigidity of these fibrin clots.[315,316] The fibrinogen molecule possesses 3 high affinity and 12 low affinity calcium binding sites.[317] One high affinity site is located on each of the two D-domains[318] whilst the other appears to be located in the central domain.[319] In circulating fibrinogen these three calcium binding sites are all

occupied.[319] Calcium ions also appear to play an important role in maintaining the structure and stability of fibrinogen, by protecting against plasmin digestion of fibrinogen and fibrin, as well as against heat and alkali denaturation.[320,321]

Interaction with proteins

The ability of fibrin(ogen) to interact with a variety of other proteins allows it to play an important regulatory role in haemostasis and fibrinolysis.

1. Thrombin

The interaction of fibrin(ogen) with thrombin via non-substrate binding sites (see figure 5b, page 68) is important in regulating thrombin activity.[82-86] Fibrin binding to thrombin localises thrombin activity to the immediate vicinity of the fibrin clot[98-100] and, importantly, fibrin bound thrombin is resistant to inhibition by circulating heparin-antithrombin III.[101,102] This allows thrombin activity to be focussed at the of site clot formation, preventing uncontrolled propagation of the thrombus.

2. Plasminogen

The interaction of plasminogen with fibrin plays an important role in regulating plasmin mediated fibrinolysis. Fibrin binding of plasminogen allows it to be more readily cleaved by t-PA.[168,169] In addition, the susceptibility of fibrin bound plasmin to inhibition by alpha-2-antiplasmin is markedly reduced compared to circulating plasminogen.[172] Thus, fibrin binding serves to localise plasminogen activation, as well as its catalytic activity, in the immediate vicinity of the fibrin clot.

3. Tissue plasminogen activator

Fibrin binding of t-PA substantially enhances its affinity for plasminogen thereby upregulating plasmin generation at the site of fibrin formation.[151]

4. Transglutaminase (factor XIIIa)

The zymogen form of factor XIII circulates as a complex bound to the D-domain of plasma fibrinogen.[322,323] Several observations suggest that specific interactions of factor XIII with fibrinogen and fibrin may regulate its activation in vivo. Fibrinogen has been shown to lower the calcium requirement for efficient activation of factor XIII,[324] and increases the rate of exposure of the active site at a given calcium concentration.[325] This "promoting" activity has been localised to the midportion of the fibrinogen Aα chain.[326] Fibrin serves as a co-factor for the thrombin mediated conversion of factor XIII to its active form factor XIIIa. This is achieved through the formation of a fibrin-factor XIII complex which has a much greater susceptibility to thrombin catalysed activation than does factor XIII alone.[327]

5. Fibronectin

Fibronectin comprises about 3-4% of a normal plasma clot.[328] This large dimeric plasma glycoprotein has an affinity for fibrinogen and, in particular, fibrin. Several types of interactions between fibronectin and fibrin(ogen) can occur, as fibronectin has both specific fibrin binding domains and a covalent cross-linking site. It can be cross-linked to fibrin by transglutaminase; this involves the α-chain of fibrin[329] and the N-terminal region of fibronectin.[330] At physiologically relevant concentrations, fibronectin incorporation into the fibrin clot enhances its elasticity.[331] Fibrin-fibronectin interactions also have important consequences at the site of wound healing. Fibrin-bound fibronectin, particularly when crosslinked, provides an excellent substratum for the migration and adhesion of fibroblasts.[332]

References

1. Henschen A, Lottspeich F. (1980) *Hematologia.* **65**, 535.
2. Henschen A. (1978) *Hoppe-Seyler's Z Physiol. Chem.* **359**, 1757.
3. Doolittle RF. (1980) In Schmidt RM (Ed) *Clinical Laboratory Science.* Vol III, section 1: Haematology, pp 3-14, CRC press.
4. Bouma H, Takagi T, Doolittle RF. (1978) *Thromb. Res.* **13**, 557.
5. Doolittle RF. (1984) *Ann. Rev. Biochem.* **53**, 195.
6. Hall CE, Slayter HS. (1959) *J. Biophys. Biochem. Cytol.* **5**, 11.
7. Fowler WE, Erikson HP. (1979) *J. Mol. Biol.* **134**, 241.
8. Williams RC. (1981) *J. Mol. Biol.* **150**, 399.
9. Blomback (1970) *Symp. Zool. Soc. London.* **27**, 167.
10. Weisel JW, Stuaffacher CV, Bullitt E, Cohen C. (1985) *Science.* **230**, 1388.
11. Williams RC. (1983) *Ann. NY Acad. Sci.* **408**, 180.
12. Weisel JW, Stauffacher CV, Bullitt E, Cohen C. (1985) *Science* **230**, 1388.
13. Rao SPS, Poojary MD, Elliott BW, Melanson LA, Oriel B, Cohen C. (1991) *J. Mol. Biol.* **222**, 89.
14. Verklich YI, Gorkun OV, Medved LV, Nieuwenhuizen W, Weisel JW. (1993) *J. Biol. Chem.* **268**, 13577.
15. Doolittle RF, Goldbaum DM, Doolittle LR. (1978) *J. Mol. Biol.* **120**,311.
16. Henschen A. (1964) *Arkiv. Kemi.* **22**, 355.
17. Blomback B, Blomback M, Henschen A, Hessel B, Iwanaga S, Woods KR. (1968) *Nature.* **218**, 130.
18. Iwanaga S, Wallen P, Grondahl NJ, Henschen A, Blomback B. (1967) *Biochim. Biophys. Acta.* **147**, 606.
19. Hoeprich PD Jnr, Doolittle RF (1983) *Biochemistry.* **22**, 2049.
20. Zhang J-Z, Redman CM. (1994) *J. Biol. Chem.* **269**, 652.
21. Zhang J-Z, Kudryk B, Redman CM. (1993) *J. Biol. Chem.* **268**, 11278.
22. Huang S, Cao Z, Davie EW. (1993) *Biochem. Biophys. Res. Commun.* **190**, 488.
23. Zhang J-Z, Redman CR. (1996) *J. Biol. Chem.* **271**, 30083.
24. Kant JA, Crabtree GR (1983) *J. Biol. Chem.* **258**, 4666.
25. Kant JA, Fornace AJ Jr, Saxe D. (1985) *Proc. Natl. Acad. Sci.* **82**, 2344.
26. Imam AM, Eaton MA, Williamson R, Humphries SE. (1983) *Nucl. Acids Res.* **11**, 7427.
27. Chung DW, Que BG, Rixon MW, Mace M Jr, Davie EW. (1983) *Biochemistry.* **22**, 3244.
28. Chung DW, Chan W-Y, Davie EW. (1983) *Biochemistry.* **22**, 3250.
29. Rixon MW, Chan W-Y, Davie EW, Chung DW.(1983) *Biochemistry.* **22**, 3237.
30. Crabtree GR, Kant JA. (1981) *J. Biol. Chem.* **256**, 9718.
31. Kant JA, Lord ST, Crabtree GR. (1983) *Proc. Natl. Acad. Sci. USA.* **80**, 3953.
32. Barnhart MI, Anderson GF. (1962) *Proc. Soc. Exp. Biol. Med.* **110**, 734.
33. Straub PW. (1963) *J. Clin. Invest.* **42**, 130.
34. Takeda Y. (1966) *J. Clin. Invest.* **45**, 103.
35. Collen D, Tytgat GN, Claeys H, PiessensR. (1972) *Br. J. Haematol.* **22**, 681.
36. Yu S, Sher B, Kudryk B, Redman CM. (1983) *J. Biol. Chem.* **258**, 13407.

37. Yu S, Kudryk B, Redman CM. (1986) In Lane DA, Henschen A, Jasani MK (eds) *Fibrinogen-Fibrin Formation and Fibrinolysis*. Vol 4, pp 3-13. Berlin: Walter de Gruyter.
38. Crabtree GR, Kant JA. (1982) *J. Biol. Chem.* **257**, 7277.
39. Kudryk B, Okada M, Redman CM, Blomback B (1982) *Eur. J. Biochem.* **125**, 673.
40. Yu S, Sher B, Kudryk B, Redman CM. (1984) *J. Biol. Chem.* **258**, 13407.
41. Yu S, Kudryk B, Redman CM. (1986) In Lane DA, Henschen A, Jasani MK (eds) In *A Scheme for the Assembly of Human Fibrinogen*. pp 665-676. New York: Walter de Gruyter.
42. Yu S, Kong V, Kudryk B, Redman CM (1987) *Thromb. Res.* **46**, 281.
43. Roy SN, Mukhopadhyay G, Redman CM. (1990) *J. Biol. Chem.* **265**, 6389.
44. Hartwig R, Danishefsky KJ. (1991) *J. Biol. Chem.* **266**, 6578.
45. Huang S, Mulvihill ER, Farrell DH, Chung DW, Davie EW. (1993) *J. Biol. Chem.* **268**, 8919.
46. Huang S, Cao Z, Davie EW. (1993) *Biochem. Biophys. Res. Comm.* **190**, 488.
47. Plant PW, Grieninger G (1986) *J. Biol. Chem.* **261**, 2331.
48. Gordon AH, Koj A. (1968) *Br. J. Exp. Pathol.* **49**, 436.
49. Fuller GM, Otto JM, Woloski BM, Mc Gary CT, Adams MA. (1985) *J. Cell Biol.* **101**, 1481.
50. Birch HE, Schreiber G (1986) *J. Biol. Chem.* **261**, 8077.
51. Otto JM, Grenett HE, Fuller GM (1987) *J. Cell Biol.* **105**, 1067.
52. Ritchie DG, Levy BA, Adams MA, Fuller GM. (1982) *Proc. Natl. Acad. Sci.* **79**, 1530.
53. Baumann H, Jahreis GP, Sauder DN, Koj A (1984) *J. Biol. Chem.* **259**, 7331.
54. Baumann H, Richards C, Gauldie J. (1987) *J. Immunol.* **139**, 4122.
55. Darlington GJ, Wilson SR, Lachman LB (1986) *J. Cell Biol.* **103**, 787.
56. Castell JV, Gomez -Lechon MJ, David M. (1988) *FEBS Letters.* **232**, 347.
57. Castell JV, Gomez -Lechon MJ, David M. (1989) *FEBS Letters.* **242**, 237.
58. Jambou RC, Snouwaert JN, Abendroth Bishop G. (1988) *Proc. Natl. Acad. Sci.* **85**, 9426.
59. Hassan JH, Chelucci C, Peschle C, Sorrentino V. (1992) *Thromb. Haemostas.* **67**, 478.
60. Franks JJ, Kirsch RE, Frith LO'C, Purves LR, Franks WT, Franks JA, Mason P, Saunders SJ. (1981) *J. Clin. Invest.* **67**, 575.
61. Davey MG, Luscher EF. (1967) *Nature.* **216**, 857.
62. Shuman MA. (1986) *Ann. NY Acad. Sci.* **485**, 228.
63. Bar-Shavit R, Kahn A, Wilner GD, Fenton II JW (1983) *Science.* **220**, 728.
64. Chen LB, Buchanan JM. (1975) *Proc. Natl. Acad. Sci.* **72**, 131.
65. Chen LB, Teng NNH, Buchanan JM. (1976) *Exp. Cell Res.* **101**, 41.
66. Mc Namara CA, Sarembok IJ, Gimple LW, Fenton JW, Coughlin SR, Owens GK. (1993) *J. Clin Invest.* **91**, 94.
67. Weksler BB, Ley CW, Jaffe EA. (1978) J. *Clin. Invest.* **62**, 923.
68. Prescott SM, Zimmerman GA, Mc Intyre. (1984) *Proc. Natl. Acad. Sci. USA.* **81**, 3534.
69. Gelehrter TD, Sznycer-Laszyk R. (1986) *J. Clin Invest.* 77, 165.

70. Daniel TO, Gibbs VC, Milfay DF, Garavoy MR, Williams LT. (1986) *J. Biol. Chem.* **261**, 9579.
71. Zimmerman GA, Mc Intyre TM, Prescott (1986) *Ann. NY Acad. Sci.* **485**, 349.
72. Suzuki K, Dahlback B, Stenflo J. (1982) *J. Biol. Chem.* **257**,6556.
73. Rapaport SI, Hjort PF, Patch MJ. (1965) *Scand. J. Clin. Lab. Invest.* **17** (Suppl), 84.
74. Schwartz ML, Pizzo SV, Hill RL, Mc Kee PA. (1973) *J. Biol. Chem.* **248**,1395.
75. Kisiel W, Canfield WM, Erikson LH, Davie EW. (1977) *Biochemistry.* **16**, 5824.
76. Vu TH, Hung DT, Wheaton VI, Coughlin SR. (1991) *Cell.* **64**, 1057.
77. Bailey K, Bettleheim FR, Lorand L, Middlebrook WR. (1951) *Nature.* **167**, 233.
78. Scheraga HA, Laskowski M Jr. (1957) *Adv. Protein Chem.* **12**, 1.
79. Blomback B. (1958) *Archiv Kemi.* **12**, 321.
80. Laurent TC, Blomback B. (1958) *Act. Chem. Scand.* **12**, 1875.
81. Blomback B, Hessel B, Hogg D, Therkildsen I. (1978) *Nature.* **275**, 501.
82. Kaminski M, Siebenlist KR, Mossesson MW. (1991) *J. Lab. Clin. Med.* **117**, 218.
83. Liu CY, Nossel HL, Kaplan KL. (1979) *J. Biol. Chem.* **254**, 10421.
84. Berliner LJ, Sugawara Y, Fenton JW II. (1985) *Biochemistry.* **24**, 7005.
85. Fenton JW II, Olson TA, Zabinski MP, Wilner GD. (1988) *Biochemistry.* **27**, 7106.
86. Wilner GD, Danitz MP, Mudd MS, Hsieh K-H, Fenton JW II. (1981) J. Lab. Clin. Med. **97**, 403.
87. Bode W, Turk D, Karshikov A. (1992) *Protein Sci.* **1**, 426.
88. Bode W, Mayr I, Baumann U, Huber R, Stone SR. (1989) *EMBO J.* 8, 3467.
89. Stubbs MT, Bode W. (1993) *Thromb. Res.* **69**, 1.
90. Noe G, Hofsteenge J, Rovelli G, Stone SR. (1988) *J. Biol. Chem.* **263**, 11729.
91. Stubbs MT, Oschkinat H, Mayr I, Huber R, Angliker H, Stone SR, Bode W. (1992) *Eur. J. Biochem.* **206**, 187.
92. Martin PD, Robertson W, Turk D, Huber R, Bode W, Edwards BF. (1992) *J. Biol. Chem.* **267**, 7911.
93. Budzynski AZ, Olexa SA, Pandya BV. (1983) *Ann. NY Acad. Sci.* **408**, 301.
94. Martinelli RA, Scheraga HA (1980) *Biochemistry* **19**, 2343.
95. Hurlet-Jensen A, Cummins HZ, Nossel HL, Liu CY. (1982) *Thromb. Res.* **27**, 419.
96. Ruf W, Bender A, Lane DA, Preissner KT, Selmayr E, Muller-Berghaus G. (1988) *Biochim. Biophys. Acta.* (1988) **965**, 169.
97. Meh DA, Siebenlist KR, Mossesson MW. (1996) *J. Biol. Chem.* **271**, 23121.
98. Al-Mondhiry HAB, Bilezikian SB, Nossel HL. (1975) *Blood.* **45**, 607.
99. Di Minno G, Martinez J, Cirillo F, Cerbone AM, Silver MJ, Colucci M, Margaglini M, Tauro R, Semeraro N, Quattrone A, Mancini M. (1991) *Arterioscler. Thromb.* **11**, 785.
100. Mossesson MW. (1993) *Semin. Thromb. Haemostas.* **19**(4), 361.
101. Hogg PJ, Jackson CM. (1989) *Proc. Natl. Acad. Sci USA.* **86**, 3619.
102. Mirshahi M, Soria J, Soria C. (1989) *Blood.* **74**, 1025.
103. Williams DO, Borer J, Braunwald E, Chesebro JH, Cohen LS, Dalen J, Dodge HT, Francis CK, Knatterud G, Ludbrook P, Markis JE, Pasamani ER, Powers E,

Rao AK, Roberts R, Ross A, Ryant T, Sobel BE, Winniford M, Zaret B. (1986) *Circulation.* **73**, 338.
104. Gold KH, Leinbach RC, Garabedian HD, Yasuda T, Johns JA, Grossbard EB, Palacios I, Collen D. (1986) *Circulation.* **73**, 347.
105. Timmis GC, Mammen EF, Ramos RG, Gordon S, Gangadharan V, Hauser AM, Westveer DC, Stewart JR. (1986) *Arch. Int. Med.* **146**, 667.
106. Chesebro JH, Knatterud G, Roberts R, Borer J, Cohen LS, Dalen J, Dodge HT, Francis Ck, Hillis D, Ludbrook P, Markis JE, Mueller H, Passamani ER, Powers ER, Rao AK, Robertson T, Ross A, Ryan TJ, Sobel BE, Willerson T, Williams DO, Braunwald E. (1987) *Circulation.* **76**, 142.
107. Greenberg CS, Achyuthhan KE, Fenton JW II. (1987) *Blood.* **69**, 867.
108. Ferry JD, (1952) *Proc. Natl. Acad. Sci. USA.* **38**, 566-569.
109. Laudano AP, Doolittle RF. (1978) *Proc. Natl. Acad. Sci. USA.* **75**, 3085.
110. Laudano AP, Doolittle RF. (1980) *Biochemistry.* **19**, 1013.
111. Pandya BV, Gariel JL, O'Brien JO, Budzynski AZ. *Biochemistry.* (1991) **30**, 162.
112. Kudryk B, Collen D, Woods KR, Blomback B. (1974) *J. Biol. Chem.* **249**, 3322.
113. Kudryk B, Reuterby J, Blomback B. (1973) *Thromb. Res.* **2**, 297.
114. Olexa SA, Budzynski AZ, (1980) *Proc. Natl. Acad. Sci. USA.* 77, 1374.
115. Olexa SA, Budzynski AZ, Doolittle RF, Cottrell BA. (1980) *Biochemistry.* **21**, 6139.
116. Shimizu A, Nagel GM, Doolittle RF. (1992) *Proc. Natl. Acad. Sci. USA.* **89**, 2888.
117. Yamazumi K, Doolittle RF. (1992) *Proc. Natl. Acad. Sci. USA.* **89**, 2893.
118. Krakow W, Endres GF, Siegel BM, Scheraga HA. (1972) *J. Mol. Biol.* **71**, 95.
119. Fowler WE, Handgan RR, Hermans J, Erikson HP. (1981). *Proc. Natl. Acad. Sci. USA.* **78**, 4872.
120. Handgan R, Mc Donagh J, Hermans J. (1993) *Ann. NY Acad. Sci.* **408**, 344.
121. Shainoff JR, Dardik BN. (1983) *Ann. NY Acad. Sci.* **408**, 254.
122. Shainoff JR, Dardik BN. (1979) *Science.* **204**, 200.
123. Mossesson MW, Siebenlist KR, Hainfeld JF, Wall JS. (1995) *J. Struct. Biol.* **115**, 88.
124. Mossesson MW, Siebenlist KR, DiOrio JP, Matsuda M, Hainfeld JF, Wall JS. (1995) *J. Clin. Invest.* **96**, 1053.
125. Siebenlist KR, Meh DA, Wall JS, Hainfeld JF, Mossesson MW. (1995) *Thromb. Haemostas.* **74**, 1113.
126. Mossesson MW, Sherry S. (1966) *Biochemistry.* **5**, 2829.
127. Hasegawa N, Sasaki S. (1990) *Thromb. Res.* **57**, 183.
128. VerklichYI, Gorkun OV, Medved LV, Niewenhuizen W, Weissel JW. (1993) *J. Biol. Chem.* **268**, 13577.
129. Gorkun OV, VerklichYI, Medved LV, Henschen A, Weissel JW. (1993) *Biochemistry.* **33**, 6986.
130. Mosesson MW. (1992) *Semin. Haematol.* **29**, 177.
131. Shimizu A, Ferry JD. (1988) *Biopolymers.* **27**, 703.
132. Shen L, Lorand L. (1983) *J. Clin. Invest.* **71**, 1336.
133. Matacic S, Loewy AG (1968) *Biochem. Biophys. Res. Commun.* **30**, 356.
134. Pissano JJ, Finlayson JS, Peyton MP. (1968) *Science.* **160**, 892.

135. Chen R, Doolittle RF. (1971) *Biochemistry.* **10**, 4486.
136. Doolittle RF. (1973) *Thromb. Diath. Haemorrh.* **54** (suppl), 155.
137. Purves LR, Purves M, Brandt W. (1987) *Biochemistry.* **26**, 4640.
138. Mossesson MW, Siebenlist KR, Meh DA, Wall JS, Hainfeld JF. (1998) *Proc. Natl. Acad. Sci.* USA. **95**, 10511.
139. Mossesson MW, Siebenlist KR, Amrami DL, DiOrio JP. (1989) *Proc. Natl. Acad. Sci.* USA. **86**, 1113.
140. Shainoff JR, Urbanic DA, DiBello PN. (1991) *J. Biol. Chem.* **166**, 6429.
141. Mc Kee PA, Mattock P, Hill RL. (1970) *Proc. Natl. Acad. Sci.* USA. **66**, 738.
142. Kamykowski GW, Mosher DF, Lorand l, Ferry JD. (1981) *Biophys. Chem.* **13**, 25.
143. Sakata Y, Aoki N. (1982) *J. Clin. Invest.* **69**, 536.
144. Dano K, Andreasen J, Grohndal-Hansen J, Kristensen P, Nielsen LS, Skriver L (1985) *Adv. Cancer Res.* **44**, 139.
145. Vassallli J-D, Baccino D, Belin D. (1985) *J. Cell Biol.* **100**, 86.
146. Blasi F, Behrendt N, Cubellis MV, Ellis V, Lund LR, Masucci MT, Moller LB, Olson DP, Pedersen N, Ploug M, Ronne E, Dano K. (1990) *Cell Diff. Dev.* **32**, 247.
147. Gething MJ, Adler B, Boose JA, Gerard RD, Madison EL, McGookey D, Meidell RS, Roman LM, Sambrook J. (1988) *Eur. Mol. Biol. Organ. J.* 7, 2731.
148. Saksela O, Rifkin D. (1988) *Ann. Rev. Cell. Biol.* **4**, 93.
149. Wiman B, Collen D. (1978) *Nature (Lond.)*, **272**, 549.
150. Niewenhuizen W. (1988) In Kluft C (Ed), *Tissue-type plasminogen activator (t-PA): Physiological and Clinical Aspects.* Vol 1, pp 171-187.CRC Press, Boca Raton.
151. Hoylaerts M, Rijken DC, Lijnen HR. (1982) *J. Biol. Chem.* **257**, 2912.
152. Voskuilen M, Vermond A, Veenaman GH, Van Boom JH, Klaasen EA, Zegers ND, Nieuwenhuizen W (1987) *J. Biol. Chem.* **262**, 5944.
153. Nieuwenhuizen W, Vermond A, Voskuilen M, Traas DW, Verheijen JH. (1983) *Biochim. Biophys. Acta.* **748**, 86.
154. Yonekawa O, Voskuilen M, Nieuwenhuizen W. (1992) *Biochem. J.* **283**, 187-191.
155. Niewenhuisen W. (1994) *Thromb. Res.* **75**, 343.
156. Schielen, WJG, Voskuilen M, Tesser GI, Nieuwenhuizen W. (1989) *Proc. Natl. Acad. Sci. USA.* **86**, 8951.
157. Schielen, WJG, Adams HPHM, van Leuven K, Voskuilen M, Tesser GI, Nieuwenhuizen W. (1991) *Blood.* 77, 2169.
158. Andreasen PA, George B, Lund LR, Riccio, Stacey SN. (1990) *Mol. Cell. Endocrinol.* **68**, 1.
159. Vassalli JD, Sappino AP, Belin D. (1991) *J Clin Invest.* **88**, 1067.
160. Werb Z, Banda MJ, Jones PA. (1980) *J. Exp. Med.* **152**, 1340.
161. Violand BN, Castellino FJ. (1976) *J. Biol. Chem.* **251**, 3906.
162. Patthy L, Trexler M, Vali Z. (1984) *FEBS Lett.* **171**, 131.
163. Sugiyama N, Iwamoto M, Abiko A. (1987) *Thromb. Res.* **47**, 459.
164. Wiman B, Lijnen HR, Collen D. (1979) *Biochim. Biophys. Acta.* **579**, 142.
165. Markus G, DePasquale JL, Wissler FC. (1978) *J. Biol. Chem.* **253**, 727.
166. Vali Z, Patthy L. (1984) *J. Biol. Chem.* **259**, 13690.

167. Castellino FJ. (1981) *Chemical Reviews.* **81**, 431.
168. Mangel W, Lin B, Ramakrishnan V. (1990) Science. **248**, 69.
169. Takada A, Sugawara Y, Takada Y. (1989) Haemostasis. **1**, 26.
170. Robbins KC, Summaria L, Hsieh B, Shah RJ. (1967) *J. Biol. Chem.* **242**, 2333.
171. Aoki N, Harpel PC. (1984) *Sem. Thromb. Haemostas.* **10**, 24.
172. Collen D. (1980) *Thromb. Haemostas.* **43**, 77.
173. Doolittle RF. (1981) *Scient. Amer.* **245**, 92.
174. Gaffney PJ. (1973) *Thromb. Res.* **2**, 201.
175. Fergusson EW, Fretto LJ, McKee PA. (1975) *J. Biol. Chem.* **250**, 7210.
176. Pizzo SV, Taylor LM, Schwartz ML, Hill RL, Mc Kee PA. (1973) *J. Biol. Chem.* **248**, 4584.
177. Pizzo SV, Taylor LM, Schwartz ML, Hill RL, Mc Kee PA. (1973) *J. Biol. Chem.* **248**, 4574.
178. Mihalyi E, Weinberg R, Towne D. (1976) *Biochemistry.* **15**, 5372.
179. Gaffney PJ, Dobos P. (1971) *FEBS Lett.***15**, 13.
180. Pizzo SV, Schwartz ML, Hill RL, McKee PA. (1972) *J. Biol. Chem.* **247**, 636.
181. Catanzaro A, Strathern J, Edgington TS. (1971) *Fed Proc., Fed. Am. Soc. Exp. Biol.* **30**, 339.
182. Purves LR, Lindsey GG, Brown G, Franks JJ. (1978) *Thromb. Res.* **12**, 473.
183. Gaffney PJ, Joe F. (1979) *Thromb. Res.* **15**, 673.
184. Edgington TS, Plow EF. (1975) *J. Biol. Chem.* **250**, 3393.
185. Catanzano A, Edgington TS. (1974) *J. Lab. Clin. Med.* **83**, 458.
186. Francis CW, Marder VJ, Martin SE. (1980) *Blood.* **56**, 456.
187. Sakharov DV, Nagelkerke JF, Rijken DC. (1995) *Circulation.* **92**, 1883.
188. Sakharov DV, Nagelkerke JF, Rijken DC. (1996) *J. Biol. Chem.* **271**, 2133.
189. Plow EF. (1980) *Biochem Biophys Acta.* **630**, 47.
190. Plow EF. (1982) *J Clin Invest.* **69**, 564.
191. Bilezikian SB, Nossel HL. (1977) *Blood.* **50**, 21.
192. Campbell EJ, Senior RM, Mc Donald JA, Cox DL. (1982) *J Clin Invest.* **70**, 845.
193. Wu K, Urano T, Ihara H, Takada Y, Fujie M, Shikimori M, Hashimoto K, Takada A. (1995) *Blood.* **86**, 1056.
194. Bach-Gansmo ET, Halvorsen S, Godal HC, Skjonsberg OH. (1995) *Thromb Res.* **80**, 153.
195. Plow EF, Edgington TS. (1975) *J. Clin. Invest.* **56**, 30.
196. Gramse M, Bingenheimer C, Havemann K. (1980) *Thromb. Res.* **19**, 201.
197. Gramse M, Bingenheimer C, Schmidt W, Egbring R, Havemann K. (1978) *J. Clin. Invest.* **61**, 1027.
198. Simon DI, Ezratty AM, Francis SA, Rennke H, Loscalzo J. (1993) *Blood.* **82**, 2414.
199. Loscalzo J. (1996) *Semin. Thromb, Haemostas.* **22**, 503.
200. Rulot H. (1904) *Arch. Int. Physiol. Biochim.* **1**, 152.
201. Opie EL. (1907) J. Exp. Med. **9**, 391.
202. Chandler AB. (1969) In Sherry S, Brinkhous KM, Genton E, Stengle JM (eds). *Thrombosis: The anatomy of a thrombus* pp 279-299. Washington DC, National Academy of Sciences.
203. Henry RL. (1965) *Thromb. Diath. Haemorrh.* **13**, 35.

204. Barnhart MI. (1965) *Fed. Proc.* **24**, 846.
205. Riddle JM, Barnhart MI. (1964) *Am. J. Pathol.* **45**, 805.
206. Paz RA, Spector WG. (1962) *J. Pathol. Bacteriol.* **84**, 85.
207. Senior RM, Skorgen WF, Griffin GL, Wilner GD. (1986) *J. Clin. Invest.* **77**, 1014.
208. Prokopowicz J. (1968) *Biochim. Biophys. Acta.* **154**, 91.
209. Prokopowicz J. (1968) *Thromb. Diath. Haemorrh.* **19**, 84.
210. Plow EF, Edgington TS. (1978) *Thromb. Res.* **12**, 653.
211. Herman G, Miescher PA. (1965) *Int. Ach. Allergy Appl. Immunol.* **27**, 346.
212. Ohlsson K, Olsson I. (1974) *Eur. J. Biochem.* **42**, 519.
213. Schmidt W, Egbring R, Haveman K. (1975) *Thromb. Res.* **6**, 315.
214. Schmidt W, Havemann K. (1977) *Hoppe Seyler's Z. Physiol. Chem.* **358**, 555.
215. Adams SA, Kelly SL, Kirsch RE, Shephard EG. (1998) *Blood Coag. Fibrinol.* **9**, 307.
216. Bach-Gansmo ET, Halvorsen S, Godal HC, Skjonsberg OH. (1996) *Thromb. Res.* **82**, 177.
217. Weitz JI, Huang AJ, Landman SL, Nicholson SC, Silverstein SC. (1987) *J. Exp Med.* **166**, 1836.
218. Barrett AJ, Salvesen. (1986) *Proteinase Inhibitors.* pp 3-661. Amsterdam, Elsevier. **CHECK**
219. Gramse M, Egbring R, Havemann K. (1984) *Hoppe Seyler's Z. Physiol. Chem.* **365**, 19.
220. Machovich R, Owen WG. (1989) *Biochemistry.* **28**, 4517.
221. Moroz LA. (1981) *Blood.* **58**, 97.
222. Drag B, Petersen LC. (1994) *Fibrinolysis.* **8**, 192.
223. Kolev K, Lerant I, Tenekejiev K, Machovich R. (1994) *J. Biol. Chem.* **269**, 17030.
224. Loike JD, Silverstein R, Wright SD, Weitz JI, Huang AJ, Silverstein SC. (1992) *Ann. NY Acad. Sci.* **667**, 163.
225. Owen CA, Campbell MA, Boukedes SS, Campbell EJ. (1997) *Amer J Physiol.* **272**, L385.
226. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. (1995) *J Cell. Biol.* **131**(3), 775.
227. Gaudin P, Berthier S, Barro C, Zaoui P, Morel F. (1997) *Eur. J. Cell Biol.* **72**, 345.
228. Salcedo R, Wasserman K, Patarroyo M. (1997) *Exp. Cell Res.* **233**, 33.
229. Bangalore N, Travis J. (1994) *Biol. Chem Hoppe Seyler.* **375**, 659.
230. Bach-Gansmo ET, Halvorsen S, Godal HC, Skjonsberg OH. (1994) *Thromb. Res.* **75**, 307.
231. Camire RM, Kalafatis M, Tracy PB. (1998) *Biochemistry.* **37**, 11896.
232. Turkington PT. (1991) *Haemostasis.* **21**, 111.
233. Turkington PT (1992) *Thromb. Res.* **67**, 147.
234. Eckle I, Seitz R, Egbring R, Kolb G, Havemann K. (1993) *Scand. J. Clin. Lab. Invest.* **52**, 281.
235. Kelly SL, Adams SA, Robson SC, Kirsch RE, Shephard EG. (1994) *Biochem. J.* **298**, 689.

236. Adams SA, Kelly SL, Kirsch RE, Robson SC, Shephard EG. (1995) *Blood Coag. Fibrinol.* **6**, 693.
237. Kelly SL (1995) A neutral protease of the neutrophil surface: Role in proteolysis of C-reactive protein and fibrinogen (PhD Thesis, University of Cape Town)
238. Adams SA (1996) Proteases of the neutrophil membrane represent an alternative fibrinolytic pathway to that mediated by plasmin (PhD Thesis, University of Cape Town).
239. Plesner T, Ploug M, Ellis V, Ronne E, Hoyer-Hansen G, Wittrup M, Lindthardt-Pedersen T, Tscherning T, Dano K, Ebbe-Hansen N. (1994) *Blood.* **83**, 808.
240. Estreicher A, Muhlhauser J, Carpentier J-L, Orci L, Vassalli J-D. (1990) *J. Cell Biol.* **111**, 783.
241. Loscalzo J. (1996) *Semin. Thromb. Haemostas.* **22**, 503.
242. Gonda SR, Schainoff JR. (1982) *Proc. Natl. Acad. Sci. USA.* **79**, 4565.
243. Carmeliet P, Schoonjans L, Kieckens L, Ream B, Degen J, Bronson R, De Vos R, van den Oord JJ, Collen D, Mulligan RC. (1994) *Nature.* **368**, 419.
244. Carmeliet P, Mulligan RC, Collen D. (1994) *J. Intern. Med.* **236**, 455.
245. Wright SD, Weitz JI, Huang AJ, Levin SM, Silverstein SC, Loike JD. (1988) *Proc. Natl. Acad. Sci. USA.* **85**, 7734.
246. Altieri DC, Plescia J, Plow EF. (1993) *J. Biol. Chem.* **268**, 1847.
247. Diamond MS, Garcia-Aguilar J, Bickford JK, Corbi AL, Springer TA. (1993) *J. Cell Biol.* **120**, 1031.
248. Loike JD, Sodeik S, Cao L, Leucona, Weitz J, Detmers PA, Wright SD, Silverstein SC. (1991) *Proc. Natl. Acad. Sci. USA.* **88**, 1044.
249. Gresham HD, Goodwin JL, Allen PN, Anderson DC, Brown EJ. (1989) *J.Cell Biol.* **108**, 1935.
250. Gresham HD, Adams SP, Brown EJ. (1992) *J. Biol. Chem.* **267**, 13895.
251. Berton G, Fumagalli L, Laudanna C, Sorio C. (1994) *J. Cell Biol.* **126**, 1111.
252. Fuortes M, Jin W, Nathan C. (1993) *J. Cell Biol.* **120**, 777.
253. Laudanna CF, Rossi F, Berton G. (1993) *Biochem. Biophys. Res. Comm.* **190**, 935.
254. Laudanna C, Miron S, Berton G, Rossi F. (1990) *Biochem. Biophys. Res. Comm.* **166**, 308.
255. Suchard SJ, Boxer LA. (1994) *J. Immunol.* **152**, 290.
256. Ugarova TP, Solovjov DA, Zhang L, Loukinov DI, Yee VC, Medved LV, Plow EF (1998) *J. Cell Biol.* **273**, 22519.
257. Higazi AA, Barghouti II, Ayesh SK, Mayer M, Matzner Y. (1994) *Inflammation.* **18**, 525.
258. Kazura JW, Wenger JD, Salata RA, Budzynski AZ, Goldsmith GH. (1989) *J. Clin. Invest.* **83**, 1916.
259. Fischer EG, Vogel P, Ziegler AS, Kirkpatrick CJ. (1991) *Haemostasis.* **21**, 141.
260. Gross TJ, Leavell KJ, Peterson MW. (1997) *Thromb. Haemostas.* **77**, 894.
261. Leavell KJ, Peterson MW, Gross TJ. (1996) *Am. J. Resp. Cell. Mol. Biol.* **14**, 53.
262. Loike JD, el Khoury J, Cao L, Richards CP, Rascoff H, Mandeville JT, Maxfield FR, Silverstein SC. (1995) *J. Exp. Med.* **81**, 1763.
263. Loike JD, Cao L, Budhu, Marcantonio EE, Elkoury J, Hoffman S, Yerdnock TA, Silverstein SC. (1999) *J. Cell Biol.* **144**, 1047.

264. Lee, L. (1962) J. Exp. Med. **116**, 611.
265. Emeis JJ, Lindeman J, Nieuwenhuizen W. (1981) *Am. J. Pathol.* **103**, 337.
266. Colvin RB, Dvorak HF. (1975) *J. Exp. Med.* **142**, 1377.
267. Sherman LA, Lee J. (1977) *J. Exp. Med.* **145**, 76.
268. Altieri DC, Mannucci PM, Capitanio AM. (1986) *J. Clin. Invest.* **78**, 968.
269. Robson, SC, Saunders R, Purves LR, De Jager C, Corrigall AV, Kirsch RE. (1993) *Blood.* **81**, 3006.
270. Robson SC, Shephard EG, Kirsch RE. (1994) *Br. J. Haem.* **86**, 322.
271. Bennet JS, Vilaire G, Cinnes DB. (1982) *J. Biol. Chem.* **257**, 8049.
272. Andrieux A, Hudry-Clergeon C, Ryckewaert J-J, Chappel A, Ginsberg MH, Plow EF, Marguerie G. (1989) *J.Biol. Chem.* **264**, 9258.
273. Farell DH, Thiagajaran P, Chung DW, Davie EW. (1992) *Proc. Natl. Acad. Sci. USA.* **89**, 10729.
274. Harrison P, Savidge GF, Cramer EM. (1990) *Br. J. Haematol.* **74**, 125.
275. Harrison P, Wilbourn, Debili N, Vainchenker W, Breton-Gorius J, Lawrie AS, Masse JM, Savidge GF, Cramer EM. (1989) *J. Clin. Invest.* **84**, 1320.
276. Handagama PJ, Bainton DF, Jacques Y, Conn M, Lazarus RA, Shuman MA. (1993) J. *Clin. Invest.* **91**, 193.
277. Handagama PJ, Scarborough RM, Shuman MA, Bainton DF. (1993) *Blood.* **82**, 135.
278. Wencel-Drake JD, Boudignon-Proudhon C, Dieter MG, Criss AB, Parise LV. (1996) *Blood.* **87**, 602.
279. Huang M-M, Lipfert L, Cunningham M, Brugge JS, Ginsberg MH, Shattil SJ. (1993) *J. Cell Biol.* **22**, 473.
280. Qi J, Kreutzer DL, Piela-Smith TH. (1997) *J. Immunol.* **158**, 1880.
281. Languino LR, Colella S, Zanetti A, Andrieux A, Ryckewaert JJ, Charon MH, Marchiso PC, Plow EF, Ginsberg MH, Marguerie G, Dejana E. (1989) *Blood.* **72**, 734.
282. Duperray A, LanguinoLR, Plescia J, Mc Dowall, Hogg N, Craig AG, Berendt AR, Altieri DC. (1997) *J. Biol. Chem.* **272**, 435.
283. Hicks RCJ, Golledge J, Mir-Hasseine R, Powell JT. (1996) *Nature.* **379**, 818.
284. Ge M, Tang G, Ryan TJ, Malik AB. (1992) *J. Clin. Invest.* **90**, 2508.
285. Weimar B, Delvos U. (1986) *Arteriosclerosis.* **6**, 139.
286. Dejana E, Colella S, Languino LR, Balconi G, Corbascio GC, Marchisio PC. (1987) *J. Cell Biol.* **104**, 1403.
287. Ribes JA, Francis CW, Wagner DD. (1987) *J. Clin. Invest.* **79**, 117.
288. Kaplan KL, Mather T, DeMarco L, Solomon S. (1989) *Arteriosclerosis.* **9**, 43.
289. Qi J, Kreutzer DL. (1995) *J. Immunol.* **155**, 867.
290. Gamble JR, Matthias LJ, Meyer LJ, Kaur P, Russ G, Faull R, Berndt MC, Vadas MA. (1993) *J. Cell Biol.* **121**, 931.
291. Dvorak HF, Harvey VS, Estrella P, Brown LF, McDonagh J, Dvorak MA. (1987) *Lab. Invest.* **57**, 673.
292. Sporn LA, Bunce LA, Francis CW. (1995) *Blood.* **86**, 1802.
293. Gray AJ, Park PW, Broekelmann TJ, Laurent GJ, Reeves JT, Stenmark KR, Mecham RP. (1995) *J. Biol. Chem.* **270**, 26602.
294. Ishida T, Tanaka K. (1982) *Atherosclerosis.* **44**, 161.
295. Ross R, Glemset JA. (1973) *Science.* **180**, 1332.

296. Shekhonin BV, Tararak EM, Samokin GP, Mitkevich OV, Mazurov AV, Vinogradov DV. (1990) *Arteriosclerosis.* **82**, 213.
297. Smith EB, Alexander KM, Massie IB. (1976) *Arteriosclerosis.* **23**, 19.
298. Bini A, Fenoglio J, Mesa-Tejada R, Kudryk B, Keplan L. (1989) *Atherosclerosis.* **9**, 109.
299. Haust MD, Wyllie JC, Moke RH. (1965) *Exp. Mol. Biol.* **4**, 205.
300. York JL, Benjamin W. (1980) *Thromb. Res.* **18**, 717.
301. Whitnack E, Beachty EH. (1982) *J. Clin Invest.* **69**, 1042.
302. Wagner B, Schmidt KH, Wagner M, Kohler W. (1986) *Zbl. Bakt. Hyg.* **A261**, 432.
303. Boschwitz JS, Timoney JF. (1994) *Microb. Pathogen.* **17**, 121.
304. Lammler C, Ding H. (1994) *Zbl. Vet. Med.* **B41**, 588.
305. Coulot P, Bouchara J-P, Renier G, Annaix V, Planchenault C, Trochin G, Chabasse D. (1994) *Infect. Immun.* **62**, 2169.
306. Lantz ML, Allen RD, Vail TA, Switalski LM, Hook M. (1991) *J. Bacteriol.* **173**, 495.
307. Annaix V, Bouchara JP, Tronchin G, Senet JM, Robert R. (1990) *FEMS Microbiol. Immunol.* **64**, 147.
308. Hawiger J, Timmons S, Strong DD, Cottrell BA, Riley M, Doolittle RF. (1982) *Biochemistry.* **21**, 1407.
309. Shen W, Steinruck H, Ljungh A. (1995) *Gut.* **36**, 401.
310. Bayer AS, Sullam PM, Ramos M, Li C, Cheung AL, Yeaman MR. (1995) *Infect. Immun.* **63**, 3634.
311. Abeck D, Johnson AP, Mensing H. (1992) *Microb. Pathogen.* **13**, 81.
312. Schmidt KH, Mann K, Cooney J, Kohler W. (1993) *FEMS Immunol. Med. Microbiol.* **7**, 135.
313. Wang H, Lottenberg, Boyle MDP. (1995) *Microb. Pathogen.* **18**, 153.
314. Mc Devitt D, Francois P, Vaudaux P, Foster TJ. (1995) *Mol. Microbiol.* **16**(5), 895.
315. Boyer MH, Shainoff JR, Ratnoff OD. (1972) *Blood.* **39**, 382.
316. Shen LL, Mc Donagh RP, Mc Donagh J, Hermans J. (1974) *Biochem. Biophys. Res. Comm.* **56**, 793.
317. Marguerie G, Chagniel G, Suscillon M. (1977) *Biochim. Biophys. Acta.* **490**, 94.
318. Nieuwenhuizen W, Vermond A, Nooijen WJ, Havercate F. (1979) *FEBS Lett.* **98**, 257.
319. Nieuwenhuizen W, van Ruijven-Vermeer, Nooijen WJ, Vermond A, Haverkate F, Hermans J. (1981) *Thromb. Res.* **22**, 653.
320. Havercate F, Timan G. (1977) *Thromb. Res.* **10**, 803.
321. Ly B, Godal HC. (1973) *Haemostasis.* **1**, 204.
322. Greenberg CS, Shuman MA. (1985) *J. Biol. Chem.* **257**, 6096.
323. Mary A, Achyuthan KE, Greenberg C. (1987) *Biochem. Biophys. Res. Comm.* **147**, 608.
324. Credo RB, Curtis CG, Lorand L. (1978) *Proc. Natl. Acad. Sci USA.* **75**, 4234.
325. Hornyak TJ, Shafer JA. (1991) *Biochemistry.* **30**, 6175.
326. Credo RB, Curtis CG, Lorand L. (1981) *Biochemistry.* **20**, 3770.
327. Naski MC, Lorand L, Shafer JA. (1991) *Biochemistry.* **30**, 934.

328. Chen AB, Amrani DL, Mosesson MW. (1977) *Biochim. Biophys. Acta.* **493**, 310.
329. Iwanaga S, Suzuki K. Hashimoto S. (1978) *Ann. NY Acad. Sci.* **312**, 56.
330. Mc Donagh RP, Mc Donagh J, Petersen TE, Thogersen HC, Skorstengaard K, Sottrup-Jensen S, Magnusson S, Dell A, Morris HR. (1981) *FEBS Lett.* **127**, 174.
331. Kamykowski GW, Mosher DF, Lorand L, Ferry JD. (1981) *Biophys. Chem.***13**, 25.
332. Grinell F, Feld M, Minter D. (1980) *Cell.* **19**, 517.

Chapter Four

Attempts to purify a 600 kDa protease

Incubation of C-reactive protein (CRP), fibrinogen and fibrin with PMA-stimulated neutrophils leads to degradation of these substrates by a membrane associated protease.[1-3] Modulation of inflammatory reactions, either by the generated products or by the proteolytically modified substrates appears to be an important consequence of the membrane associated proteolytic activity on these substrates. In the case of CRP, the generated peptides inhibit neutrophil function,[4] whilst fibrinogenolysis generates non-clottable fibrinogen which is proposed to limit the deposition of fibrin at sites of inflammation.[2] The synergistic activity of this membrane associated proteolytic activity with plasmin has been shown to aid fibrin dissolution.[3]

The products generated from CRP, fibrinogen and fibrin by the neutrophil membrane associated protease have been reported to have distinctly different apparent molecular mass values from those produced by neutrophil granule proteases, pure human neutrophil elastase (HNE) or plasmin.[1-3] This protease has been shown to be completely inhibited by the serine protease inhibitor PMSF (phenylmethylsulphonyl fluoride), but resistant to several inhibitors of azurophil granule serine proteases.[2,3] Partial release of this protease into the extracellular medium could be achieved with a dose of PMA that does not cause azurophil granule release.[1-3] Association of this protease with neutrophil cytoskeleton fractions led to the suggestion that it is a submembrane protease localised at sites of interaction of the cytoskeleton with the membrane.[1,2]

This protease was shown by size exclusion chromatography to be 600 kDa in size[1] and to resolve into subunits of 200 kDa, 316 kDa, 398 kDa and 501 kDa in size when subjected to SDS-PAGE.[1-3] Whilst the 600 kDa protease appeared to be distinct from other known neutrophil proteases on the basis of its molecular weight, inhibitor profiles and proteolytic products, it was yet to be purified from crude

extracts. As a result, its structural and catalytic properties remained to be elucidated.

The purpose of this study was to purify and further characterise this novel membrane associated protease. Attempts by others to purify this protease, involved a variety of column chromatographic techniques, but had proved unsuccessful (personal communication Sharon Kelly). The present study explored new avenues whereby the membrane associated protease could be purified. Three different approaches to the purification and characterisation of this protease are described in this chapter. Whilst unsuccessful and, in many instances incomplete, the collective conclusions drawn from these purification attempts clearly suggested a need to identify, more carefully, the nature of the membrane associated protease responsible for the degradation of CRP, fibrinogen and fibrin.

Methods

Chemicals and reagents

See *Appendix 1*, pages 225-226

Preparation and iodination of fibrinogen

Fibrinogen, isolated by ammonium sulphate precipitation of plasma obtained from heparinised blood donated by healthy laboratory workers,[5] migrated as a single band corresponding to an apparent molecular mass of 340 kDa when analysed by SDS-PAGE (non-reduced). The preparation contained a trace amount of factor XIII. The clottability of fibrinogen prepared in this way was 97%. Fibrinogen was iodinated using iodogen as an oxidising agent and 0.5 μCi [^{125}I]-Na/μg fibrinogen.[6] Iodinated fibrinogen had a specific activity of 0.5μCi/μg fibrinogen and showed no evidence of degradation when subjected to 5-20% SDS-PAGE and autoradiography (400 000 cpm/track).

Preparation of CRP

CRP was prepared from a pool of acute phase sera obtained from patients 48 hours post surgery. Informed consent and ethics committee approval was obtained. CRP was isolated from serum via calcium-dependent phosphorylethanolamine affinity chromatography and Sephacryl S-200 gel filtration.[7] Purified CRP migrated as a single band with an apparent molecular mass of 24 kDa when subjected to 12% SDS-PAGE (reduced).

Isolation of neutrophils

Neutrophils were isolated from heparinised blood (donated by healthy laboratory workers) by Ficoll-Hypaque centrifugation

followed by dextran sedimentation and hypotonic lysis of erythrocytes.[8] This procedure yields neutrophil preparations with a purity and viability of greater than 95% as determined by Wright-Giemsa staining and trypan blue exclusion respectively. Purified neutrophils were resuspended in either phosphate-buffered saline (PBS) or Hanks balanced salt solution (HBSS). Neutrophils were used immediately following purification.

Preparation of neutrophil conditioned medium

For the preparation of neutrophil conditioned medium, neutrophils were resuspended in HBSS at 10 x 10^6 cells/ml and warmed to 37°C for 15 minutes. Cells were then stimulated with phorbol 12-myristate 13-acetate (PMA) at a final concentration of 5 or 10ng/ml at 37°C for 20 minutes. Cells were gently resuspended every 5 minutes to prevent aggregation. Following centrifugation (2000rpm, 3 minutes, 4°C) the cell free supernatant was collected and used immediately as the neutrophil conditioned medium. When necessary, conditioned media were also prepared from non-stimulated neutrophils as well as neutrophils stimulated with high dose PMA (150 ng/ml).

Preparation of neutrophil cytoskeleton

Neutrophil cytoskeletons were isolated by lysing neutrophils (20 x 10^6/ml) for 10 minutes on ice in lysis buffer (50mM Tris-HCl, pH 7.5, containing 160mM KCl, 10mM EDTA and 1% (v/v) Triton X-100).[9] The triton insoluble cytoskeletons were pelleted by centrifuging at 12000g for 10 minutes at 4°C, and then washed in the lysis buffer without Triton X-100. Following 3 washing steps the proteins of the neutrophil cytoskeletal pellet were solubilised in 10 mM Tris-HCl (pH 7.4) containing 1M NaCl, for 1 hour on ice. Insoluble protein was removed by microfuging at 12000g for 5 minutes at 4°C. The supernatant (1-2ml) was then dialysed against PBS at 4°C for 20 hours (3 x 500ml), and stored at 4°C until use. When necessary, concentrated preparations were made by dialysing the supernatant against dH_2O for 1 hour (3 x 500ml) instead of PBS, after which it was lyophilised and stored at -70°C until further use.

Preparations of crude solubilised membranes

Crude preparations containing solubilised neutrophil membranes were used as a source of the membrane protease during purification attempts.[10] Isolated neutrophils were resuspended in HBSS at 30 x 10^6 PMN/ml and warmed to 37°C for 10 minutes prior to stimulation with PMA (5ng/ml). Following incubation at 37°C for a further 5 minutes, cells were centrifuged at 2000rpm for 3 minutes at 4°C. The supernatant was removed and the neutrophils were then resuspended in an equal volume of HBSS containing 25μM p-nitrophenyl-p-guanidinobenzoate (NPGB), and divided into 500μl aliquots in eppendorf tubes. Cells were microfuged for 10 seconds after which the supernatant was removed, and cells resuspended in 500μl cell lysis buffer (Tris 50mM, KCl 160mM, $CaCl_2$ 1mM, $MgCl_2$ 1mM, NPGB 25μM, pepstatin 2μM, leupeptin 2μM, octylglucoside 20 mM, pH=7.4). The cells were maintained in lysis buffer at 4°C for 1 hour after which they were microfuged for 5 minutes. The supernatant was then removed and used as a crude source of the membrane protease for subsequent purification attempts.

Affinity chromatography

Preparation of matrices

Cyanogen bromide activated Sepharose 4B

10mg fibrinogen in PBS was coupled to 3.5ml of cyanogen bromide (CNBr) activated sepharose 4B beads according to manufacturers instructions. The coupling efficiency, as determined by measuring the OD_{280} of the supernatant after coupling, was found to be 95%. Excess active groups were blocked with 1M ethanolamine for 2 hours at room temperature. Uncoupled ligand was then removed by 5 alternating washes with low pH (0.1M sodium acetate, 1M NaCl, pH=4.0) and high pH (Tris 0.025M, 1M NaCl, pH=8.5) buffers. The fibrinogen-sepharose beads were then washed thoroughly with PBS and stored at 4°C in PBS containing 0.02% NaN_3. A similar

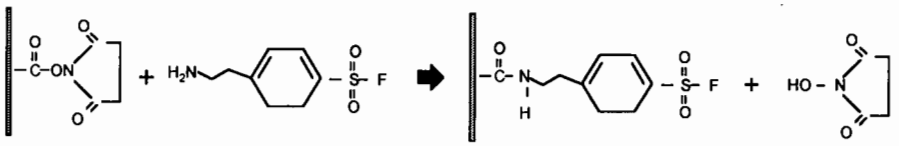
procedure was followed for the coupling of CRP (17.5mg/ml) to CNBr sepharose 4B (2.5ml) and a coupling efficiency of approximately 90% was obtained.

Tosyl-activated Dynabeads®

Dynabeads® M-280 Tosylactivated are superparamagnetic polystyrene beads activated with p-sulphonyl chloride treatment for chemical binding to proteins. Fibrinogen (0.5mg/ml) was coupled to 20mg Dynabeads® according to manufacturers instructions. The coupling efficiency, as determined by measuring the OD_{280} in the supernatant after coupling, was 86%. Protein coupled beads were washed with PBS (2 x 10ml) and then acetate buffer (0.1M sodium acetate, 1M NaCl, pH=4) to remove non-specifically bound ligand. Monitoring of the supernatant (OD_{280}) revealed that minimal protein was removed during this step. Beads were then washed with PBS/0.1% BSA (3 x 10 minutes at room temperature, 1 x 30 minute at room temperature, and overnight at 4°C) prior to use. CRP (1mg/ml) was coupled to 25mg Dynabeads, as described above, with a coupling efficiency of 50%.

Affigel 10

Affigel 10 is an N-hydroxysuccinamide ester of a derivatised crosslinked agarose bead support which contains a 10 atom spacer arm. One millilitre of Affigel 10 was coupled to 0.2mg/ml of the serine protease inhibitor, AEBSF ([2-aminoethyl]-benzenesulphonyl fluoride) according to manufacturers instructions. As AEBSF absorbs at 280nm, the coupling efficiency could be determined as described above. Interference by released N-hydroxysuccinamide ester was eliminated the addition of 1N HCl to a pH of 2.5 prior to measuring OD_{280}. The coupling efficiency was found to be 64%. Excess active groups were then blocked with 1M ethanolamine buffer, pH=8. The gel was then equilibrated in PBS prior to use.



The coupling of AEBSF to Affigel 10. *The amino group of AEBSF displaces the N-hydroxysuccinamide (NHS) group of the NHS ester forming a stable amide bond.*

Affinity chromatography

These affinity matrices were used to isolate the CRP and fibrinogen degrading activity from neutrophil conditioned medium, soluble cytoskeleton proteins, and crude preparations of soluble membrane proteins. Protease preparations were exposed to the affinity matrices either by circulation through a column packed with matrix, or through mixing the protease source with an aliquot of matrix in an eppendorf tube. Experiments were performed at 4°C to limit proteolytic activity during protease-substrate interactions and incubation times with the matrix were varied in order to optimise binding of protease. Binding of the proteolytic activity to the affinity matrix was detected by a loss of [^{125}I]-labelled fibrinogen degrading activity in crude extracts following incubation with the matrix.* For this, [^{125}I]-labelled fibrinogen was incubated (1-3 hours, 37°C) with the relevant protease sources before and after incubation with the affinity matrix. [^{125}I]-labelled fibrinogen degradation was measured by precipitating duplicate 50µl aliquots of the reaction mixture with TCA (10% final concentration) and quantifying the TCA soluble peptide formation. The concentration of [^{125}I]-labelled fibrinogen used in each degradation assay was chosen such that the crude enzyme preparations, before exposure to affinity matrices, generated approximately 30% TCA soluble [^{125}I]-labelled fibrinogen peptides at 2 hours. When a decrease in the [^{125}I]-labelled fibrinogen

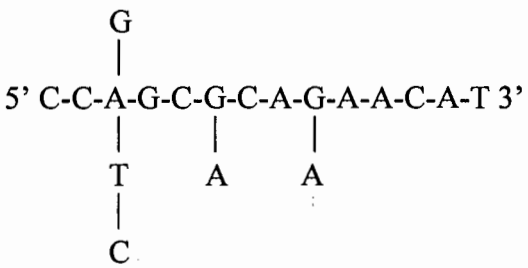
* The quantity of substrate linked to matrices was always in excess of protease levels in the crude preparations, allowing for adsorption to the column to be monitored by loss of proteolytic activity.

degrading activity of the crude extract was detected, bound material was eluted from the matrix using 4M NaCl and assayed for [^{125}I]-labelled fibrinogen degrading activity.

Molecular biology

Oligonucleotide probes

A short synthetic oligodeoxynucleotide probe, coding for a highly conserved region near the active site in a variety of different serine proteases, was used to screen a human bone marrow cDNA library. This strategy was based on previous studies in which this probe had been used to isolate a variety of serine proteases including factor IX, prothrombin, complement C1r and hepsin, from hepatocyte cDNA libraries.[11,12] A mixture of tetradecadeoxynucleotides was synthesised by Genosys according to the following sequence:



Bone marrow cDNA library

As neutrophil serine proteases are synthesised in neutrophil precursors, but not in mature neutrophils, a human bone marrow rather than a neutrophil cDNA library was screened for serine protease cDNA. This cDNA library, contained within the λDR2 expression vector, was obtained from Clontech.

[γ-^{32}P]ATP labelling of oligonucleotide probe

Oligonucleotide probes were 5' end labelled with [γ-^{32}P]ATP using T4 polynucleotide kinase. Forty pmole of oligonucleotide and 80 pmoles [γ-^{32}P]ATP (in 50mM Tris-HCl pH=7.6, 10mM $MgCl_2$, 10mM 2-mercaptoethanol) were incubated with T4 polynucleotide kinase (10U) for 30 minutes at 37°C. Radiolabelled oligonucleotide was separated from unbound [γ-^{32}P]ATP by sephadex PD-50 column chromatography. Fractions were collected, radioactivity measured using a β-counter, and peak fractions pooled and stored at -20°C until used during hybridisation procedures.

Preparation of bacterial cultures

Bacterial cultures were prepared under a laminar flow hood. A wire loop was flamed until red hot and following cooling was used to loop up a single colony of E. coli, strain K802. The loop was placed in an eppendorf tube containing 500µl of Luria broth (tryptone 1%, yeast 0.5%, NaCl 1%, pH=7.0) and agitated gently, prior to transfer of the contents of the tube into a flask containing Luria Broth (+ 10mM $MgSO_4$). The flask was incubated overnight in a 37°C shaking incubator set at 200 rpm. Following centrifugation of the overnight culture at 2500 rpm for 10 minutes at 4°C, the pellet was resuspended and diluted with SM buffer (50mM Tris-HCl, 0.1M NaCl, 10mM $MgSO_4$, 0.01% gelatin, pH=7.5) until the OD_{600} measured ±0.5.

Preparation of plates and top agar

Luria agar (tryptone 1%, yeast 0.5%, NaCl 1% and agar 1.5%) was autoclaved and allowed to cool before adding $MgSO_4$ (10mM final concentration). The agar was then poured into 150mm diameter plates which were stored at 4°C until use.

Plating of phages

Top agar was melted and then maintained in a liquid state in a 55°C water bath until required. Five microlitres of the working bacteriophage stock solution* was added to an eppendorf tube containing 400µl of E. coli (OD_{600} = 0.5) and incubated at 37°C for 15 minutes, with gentle agitation every 5 minutes. Top agar (7ml) was removed from the 55°C water bath, and $MgSO_4$ was added to achieve a final concentration of 10mM. After cooling the top agar to approximately 37°C, the contents of an eppendorf were added to it, mixing briefly but thoroughly. The top agar was then poured onto an agar plate (which had been equilibrated to 37°C) with tilting and swirling to ensure an even spread. After solidification (15 minutes), the plates were incubated at 37°C. Phage colonies appeared as small translucent plaques due to their lysis of transfected E. coli after approximately 6-8 hours.

Plaque lifts

Once plaques were clearly visible they were ready to be lifted onto Hybond-N^{TM} nylon membranes (Amersham) for subsequent hybridisation. Plates were kept at 4°C for at least 1 hour prior to plaque lifts to ensure that top agar was solidified. Membranes were placed on top of the agar for 60 seconds during which time the plates and membranes were marked in 3 assymetric points to provide a record of the position of the filter relative to the plate. Membranes were then removed and placed plaque side up in denaturing solution (1.5M NaCl, 2% w/v NaOH) for 30 seconds. Membranes were then submerged in this solution for a further 60 seconds before immersing

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* The concentration of the working stock was determined by titering the original bacteriophage stock solution. Serial dilutions of the phage stock were made (from 10^{-1} to 10^{-6}). E. coli were transfected as described above, and each dilution was plated). After 8 hours the number of plaques were counted, and the titre of the phage stock was calculated using the following equation: **Colony forming units (cfu) /ml = (# plaques x dilution factor x 10^3µl/ml) / µl used**. From this titre it was calculated that 5µl of the 10^{-3} dilution produced approximately 5000 plaques/plate. The 10^{-3} diluted phage solution was thus used as the working stock in all subsequent experiments.

in neutralising solution (1.5M NaCl, 0.5M Tris-HCl, EDTA 1mM, pH=7.2) for 5 minutes. Thereafter membranes were rinsed with 3 x SSC (20 x SSC stock: 3M NaCl, 0.3M sodium citrate, pH=7.0) and placed on filter paper where they were air dried. Once this process was completed, a second membrane was placed on the same plate, marked at the same location and subjected to the same process as described above. Membranes were placed in a UV crosslinker for 12 seconds to fix the phage DNA to the membrane. In general 5 plates at a time (and thus 10 membranes) were prepared.

Hybridisation using oligonucleotide probes

Membranes were incubated in 40ml prehybridisation solution (6 x SSC, 1 x Denhardt's solution, 100μg/ml salmon sperm DNA, 0.5% SDS and 50% formamide) at 42°C for 90 minutes. The oligonucleotide probe was heated to 100°C for 2 minutes (to release any secondary structure that may have formed) and then chilled quickly on ice. The oligonucleotide probe was then added at a minimum of 1 x 10^6 cpm/ml and hybridisation was allowed to continue for 24 hours at 30°C. Membranes were then subjected to a series of 10 minute washing steps of increasing stringency as follows: (1) 6 x SSC - room T° (2) 3 x SSC- room T° (3) 1 x SSC- room T° (4) Repeat 1 x SSC- room T° (5) 1 x SSC + 0.1% SDS- room T° (6) 0.1% SSC + 0.1% SDS- room T° (7) 0.1% SSC + 0.1% SDS at 37°C. After each washing step the radioactivity of the membranes was monitored with a Geiger counter and washes were continued until the backround radioactivity appeared to be sufficiently low. A number of the hybridisation conditions were varied over the course of several experiments in an attempt to optimise hybridisation of the probe. These included the temperature and duration of hybridisation, as well as the stringency of washes. Following washing steps, membranes were dried, taped onto a piece of filter paper placed within an X-ray cassette and marked so that developed films could later be accurately superimposed on membranes. Membranes were then subjected to autoradiography, the time of exposure varying from 1-4 days depending on the intensity of the signal. Once developed, X-ray films could be positioned such that duplicate membranes could

be compared. Any strong signal occurring in the same position on both of the duplicate filters suggested the presence of a hybridising clone.

Electrophoretic transblottting

Neutrophil cytoskeleton and conditioned medium preparations were prepared as described above. Following lyophilisation, preparations were reconstituted in dH_2O at 1/10 of the original volume, dialysed for 1 hour at 4°C against 3 changes of dH_2O after which ½ volume of 3 x concentrated SDS sample buffer (0.012 M Tris-HCl [pH 6.8] with 4% (w/v) SDS, and 24% (v/v) glycerol) was added and the mixture boiled. Samples were then analysed by 3-13% SDS-PAGE (non-reduced) according to the method of Laemmli.[13] Following electrophoresis, the gels were subjected to silver staining using a silver staining kit (Amersham) according to manufacturers instructions. Molecular weight markers ranging in molecular weight from 14 to 200 kDa (Amersham) were used to identify the apparent molecular weights of proteins of interest. When required the separated proteins were electrophoretically blotted onto polyvinylidene difluoride (PVDF) protein sequencing membrane as described previously.[14] As the subunits of the 600 kDa membrane associated protease are not detected by coomassie blue staining, and proteins on PVDF membranes cannot be silver stained, protein transfer was monitored by the disappearance of silver stained bands from polyacrylamide gels following transblotting.

Results

Affinity chromatography

Attempts to purify the 600 kDa protease using affinity chromatography utilised three sources of protease, including the conditioned medium of PMA (10ng/ml) stimulated neutrophils, soluble cytoskeleton proteins and soluble membrane proteins.

Substrate chromatography

Two substrates for this protease, namely fibrinogen and CRP, were used as affinity ligands in attempts to purify the 600 kDa protease. The substrates were coupled to either agarose (CNBr activated sepharose 4B) or tosyl-activated superparamagnetic polystyrene beads (Dynabeads®). The extent to which these matrices could extract the protease from crude preparations was determined by measuring [^{125}I]-labelled fibrinogen degrading activity before and after exposure to these matrices.

Neither CRP-sepharose nor fibrinogen-sepharose were able to extract proteolytic activity from crude preparations of either neutrophil membrane, cytoskeleton or conditioned medium. The mode of exposure of protease to the matrix (i.e. circulating through a column versus mixing in a test tube) and the duration of exposure (2-20 hours at 4°C) did not influence protease binding. In one experiment there was a 50% decrease in the fibrinogen degrading activity of a cytoskeleton preparation following exposure to CRP-sepharose for 20 hours at 4°C. However, subsequent attempts to elute proteolytic activity from the matrix using NaCl concentrations as high as 4M were unsuccessful.

Similarly, fibrinogen and CRP linked to Dynabeads, failed to extract proteolytic activity from these enzyme sources.

Inhibitor chromatography

In contrast to substrate linked matrices, AEBSF-linked affigel 10 was found to cause profound depletion of the fibrinogenolytic activity from cytoskeleton preparations containing the 600 kDa protease. (No attempt was made to isolate the proteolytic activity from membrane preparations and conditioned medium using this matrix). The [^{125}I]-labelled fibrinogen degrading activity of the neutrophil cytoskeleton was reduced by 94% following exposure to AEBSF-affigel 10. An attempt was not made to elute this protease from the beads as the interaction between serine proteases and AEBSF is irreversible.* To analyse the protein associated with the AEBSF-affigel 10 matrix, the beads were boiled in SDS sample buffer, and solubilised protein subjected to SDS-PAGE and silver staining.

No staining was observed in the areas of the gel previously reported to contain the proteolytically active subunits of the 600 kDa protease, (namely 200 kDa, 316 kDa, 398 kDa and 501 kDa),[1-3] (see figure 1, track 1). Instead there appeared to be two major areas of staining, one at approximately 30 kDa (which appeared to comprise 3 thin bands) and one between the 69 and 97 kDa molecular weight markers. Analysis of the composition of the cytoskeleton preparation before and after exposure to AEBSF-affigel 10 suggested a relative decrease in the intensity of bands at ±30 kDa and ±14-21 kDa (figure 1, tracks 2 and 3). The high molecular weight subunits of the 600 kDa protease were not visible on silver stained gels either before or after exposure to the matrix. In the context of earlier reports attributing 95% of the proteolytic activity of the cytoskeleton preparation to a 600 kDa protease,[1] the binding of low molecular weight proteins to the inhibitor linked matrix was thought, at the time, to represent non-specific binding and was not pursued further.

* Whilst it would not be possible to elute inhibitor bound protease, this technique was still considered to have potential applications provided sufficient quantities of purified 600 kDa protease could be extracted. A protease inhibitor complex could, for example, be used for the generation of antibodies.

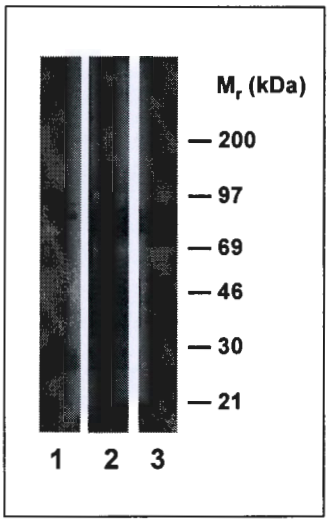


***Figure 1**. A cytoskeleton preparation (from 25 x 10^6 neutrophils) was mixed with 200µl of packed AEBSF-affigel 10 beads in a final volume of 500µl, and rolled overnight at 4°C. The beads were then allowed to settle and an aliquot of the supernatant collected. Beads were then washed and solubilised in an equal volume of 3 x SDS sample buffer and boiled, after which the beads were microfuged and the supernatant analysed by SDS-PAGE and silver staining (Track 1). The cytoskeleton preparations before and after exposure to AEBSF-affigel 10 are shown in tracks 2 and 3 respectively.*

Molecular Biology

An alternative approach, using molecular biology, was also initiated in an attempt to purify and characterise the 600 kDa protease. A strategy was chosen that involved the screening of a human bone marrow cDNA library with a short synthetic oligodeoxynucleotide probe encoding a highly conserved region near the catalytic site of a variety of serine proteases.[11,12]

Early attempts at screening this library failed to detect clones which hybridised with this oligonucleotide probe, leading to a series of experiments aimed at optimising hybridisation conditions. Concurrently with this a number of important observations were made which led to a re-evaluation of whether the protease was in fact composed of subunits with apparent molecular mass values of larger than 200 kDa. The outcome of these experiments indicated that an attempt to identify the protease using molecular biology was no longer necessary (see below).

Electrophoretic transblotting

Attempts to electrophoretically transblot the membrane associated protease were undertaken with two purposes in mind: (1) to obtain an N-terminal amino acid sequence and (2) to generate antibodies to transblotted protein.

This protease has been reported to separate into four distinct subunits when membrane, cytoskeleton and conditioned medium fractions from [^{125}I]-labelled neutrophils are subjected to SDS-PAGE and autoradiography.[1] These subunits were shown to contain the proteolytic activity of the membrane associated protease.[1-3] Whilst these subunits cannot be detected using coomassie blue staining,[1] silver staining had been successful in detecting several proteins with apparent molecular mass values greater than 200 kDa, in particular a strongly stained band thought to represent the 316 kDa subunit of this protease (personal communication).

For the present study, preparations of soluble cytoskeleton proteins, and later conditioned medium proteins, were used as sources of protease. Concentrating steps were required to ensure that sufficient protein could be detected in SDS-polyacrylamide gels. This was achieved by lyophilisation and reconstitution in 1/10 of the original volume (see *Methods*) prior to electrophoresis. Solubility problems were frequently encountered, even after the addition of SDS sample buffer. Following electrophoresis, transfer of a high molecular weight band in the 300 kDa region of the gel was successful (figure 2). However repeats of this experiment with other batches of cytoskeleton preparation

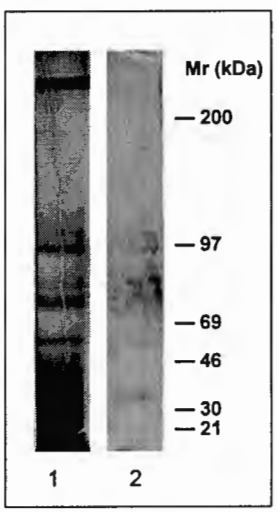


Figure 2. Electrophoretic transfer of ± 300 kDa band. *Two aliquots of cytoskeleton proteins from 100 x 10^6 neutrophils were subjected to SDS-PAGE (3-13%). Track 1 was silver stained while track 2 was transblotted onto PVDF membranes prior to silver staining. The successful transfer is suggested by the absence of staining in the gel following transblotting.*

indicated poor reproducibility in the position to which proteins with apparent molecular mass values of greater than 200 kDa migrated. Similar irreproducibility in proteins migrating with apparent molecular mass values of greater than 200 kDa was encountered when the conditioned medium was used as the source of protease (see figure 3).

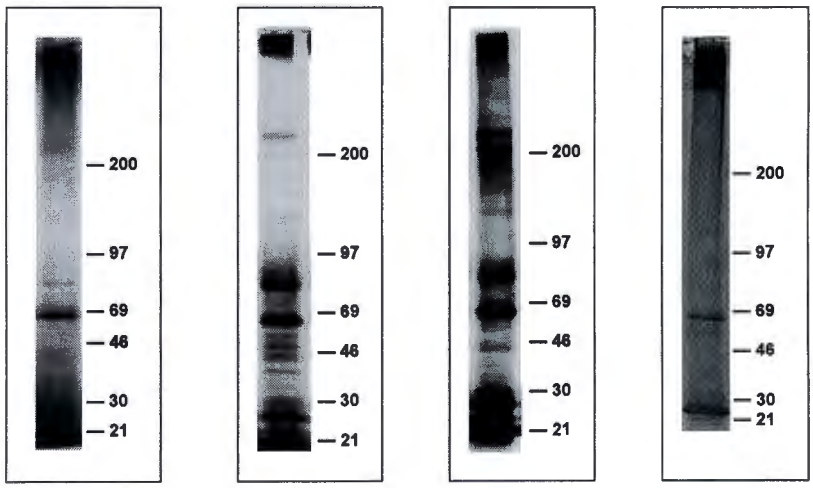


Figure 3. Variable appearance of conditioned medium preparations on SDS-PAGE. *Four different preparations of conditioned medium (1-4) had different appearances when subjected to 3-13% SDS-PAGE and silver staining. In all gels the top of the gel was darkly stained. The variable appearance of bands above 200kDa can be seen.*

When present, the intensity of bands with apparent molecular mass values of greater than 200 kDa were too low to suggest that they could be used for either protein sequencing or antibody generation. In an attempt to increase the concentration of the protease relative to the other constituents of the conditioned medium, neutrophils were stimulated with an increased dose of PMA (150ng/ml). Although this dose of PMA is known to induce azurophil granule release,[15,16] the protease of interest would subsequently be separated from the lower molecular weight granule proteases by electrophoresis, thereby preventing unwanted contamination with granule proteases. For

comparative purposes, two conditioned media were generated, one using low dose (10ng/ml) PMA (reported not to release azurophil granule proteases),[1,17] and one using PMA at 150ng/ml, and these were subjected to SDS-PAGE and silver staining (Figure 4). Unexpectedly, the electrophoretic profile of the conditioned medium generated by *high* dose PMA appeared extremely similar (albeit more concentrated) to that generated by *low* dose PMA. As high dose PMA is known to induce neutrophil release of azurophil granules, this finding suggested that conditioned medium generated by neutrophils stimulated with low dose PMA might contain azurophil granule proteases. All further attempts to purify and characterise the membrane associated protease were suspended whilst this possibility was investigated.

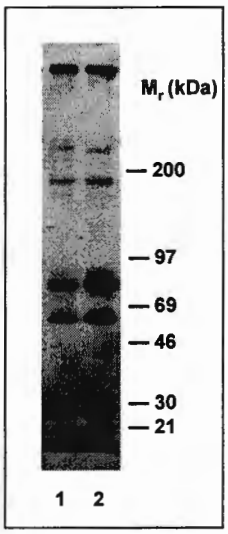


Figure 4. *Conditioned media were prepared from neutrophils (20 x 10^6/ml) stimulated with either low (10ng/ml) or high (150ng/ml) dose PMA. Following lyophilisation conditioned media were reconstituted in equal volumes and subjected to 3-13% SDS-PAGE. Track 1 shows conditioned medium generated by low dose PMA, whilst track 2 shows conditioned medium generated by high dose PMA.*

Discussion

Attempts to purify and further characterise the 600 kDa membrane associated protease utilised three different approaches - affinity chromatography, molecular biology and electrophoretic transblotting.

Affinity chromatography, using fibrinogen- and CRP-linked matrices, proved unsuccessful in extracting proteolytic activity from crude preparations containing the membrane associated protease. This may have been due to proteolytic cleavage of substrates releasing protease from the matrix, although this possibility was not investigated.

A matrix linked to the serine protease inhibitor AEBSF was able to extract proteolytic activity from preparations of soluble cytoskeleton proteins. However, SDS-PAGE analysis of the matrix bound material suggested that the 600 kDa protease had not been extracted. Instead, a broad band was observed in 30 kDa region of the gel as well as a narrow band in the 69-97 kDa region. These bands were thought at the time to represent non-specific binding to the agarose matrix, and the significance of this observation was not realised until later (see discussion, Chapter 5).

The purification of this protease was also attempted via the molecular biological route. This was stimulated by an earlier study which had used a similar approach to identify the cDNA encoding a novel serine protease in hepatocyte cDNA libraries.[11,12] The aim was to identify clones hybridising to this probe and, through DNA sequencing, to identify those clones encoding novel serine proteases. It was hoped that these could be expressed in a mammalian expression vector system, and that clones expressing the novel 600 kDa protease could be identified either immunologically (attempts were underway at the time to generate an antibody to this protease) or functionally (using indices such as substrate preference, nature of degradation products and inhibitor profiles). Finally, with the aid of molecular biological manipulations (such as the introduction of an affinity tag), it was hoped that this protease could be purified and further characterised.

Early attempts were unsuccessful in detecting clones hybridising to the oligonucleotide probe, resulting in a series of experiments aimed at optimising hybridisation conditions. At this time it became apparent from concurrent biochemical studies that the nature to the membrane associated protease needed to be re-evaluated. These studies involved attempts at electrophoretically transblotting the 316 kDa subunit of this protease. The purpose was to obtain an N-terminal amino acid sequence of this subunit and to use it for the generation of antibodies against the membrane associated protease. Although proteins with apparent molecular mass values of greater than 200 kDa could be successfully transblotted from cytoskeleton preparations, the presence of such proteins was irreproducible and the concentration too low for raising antibodies. Concentrating steps, which were required for the detection of these bands, resulted in solubility problems, and there was a tendency for large aggregates of insoluble material to form following reconstitution of lyophilised material. These processes may have accounted for the inconsistent staining of areas of the gel previously reported to contain the subunits of the 600 kDa protease. This raised the possibility that the previously documented subunits of the 600 kDa protease might, in fact, be aggregates of other proteases.

Several recent studies suggested that HNE might be a candidate protease. Neutrophils stimulated with FMLP, IL-8 or high dose PMA were reported to release HNE which could bind to the neutrophil membrane and remain proteolytically active.[15,16,18] Moreover a recent study reported the time dependent expression of HNE on the membrane of neutrophils stimulated with 0.8 nM (0.4 ng/ml) PMA.[19] The existence of both secreted and membrane bound forms of HNE suggested that HNE could be present in both the conditioned medium and membrane preparations used as a source of the 600 kDa protease. It appeared, however, that this possibility had been excluded as several lines of evidence suggested that the proteolytic activity of the 600 kDa protease was distinct from that of HNE.[1-3]

1. The 600 kDa protease was reported to generate fibrinogen and fibrin degradation products with distinctly different apparent molecular mass values from those produced by purified HNE.[2,3]
2. This protease was reported to be completely resistant to two

chloromethyl ketone inhibitors of HNE whilst demonstrating only partial sensitivity to a third.[2,3]

3. High performance liquid chromatography revealed that HNE digestion of fibrinogen produced many peptides with retention times different from those produced by the conditioned medium from PMA stimulated neutrophils. In addition, several peptides generated by the neutrophil conditioned medium were reported to still possess HNE sensitive sites.[2]
4. Amino acid sequencing of fibrinogen peptides produced by limited proteolysis by the conditioned medium suggested that fibrinolysis occurs in the N-terminal region of the Aα chain and the C-terminal regions of the Bβ and γ chains.[2] This was reported to be contrary to fibrinogenolysis by neutrophil lysates and purified HNE which cleave all three chains of fibrinogen from the N-terminus.[2]
5. The dose of PMA which was used to induce release of the 600 kDa protease into the conditioned medium was reported not to induce the release of azurophil granules.[1,17]

However, the observation, made during the present study, that the conditioned media generated by neutrophils stimulated with low (10ng/ml) and high (150ng/ml) dose PMA had similar electrophoretic profiles, suggested that low dose PMA might in fact induce release of azurophil granules and thus HNE. An additional observation made during an unrelated study (data presented in appendix 3, page 230), added to concerns that HNE might contribute to the proteolytic activity of the conditioned medium. In this study the conditioned medium was noted to degrade plasminogen, generating products which appeared remarkably similar to those generated by purified HNE. Thus, despite strong evidence to the contrary, several findings suggested the need to exclude HNE as a source of the proteolytic activity previously ascribed to a 600 kDa membrane associated protease.

As the molecular biology study represented a considerable undertaking in terms of time and expense, a more careful characterisation of the membrane associated proteolytic was essential before continuing along this line. This provided the stimulus for the study presented in the following chapter.

References

1. Shephard EG, Kelly SL, Anderson R, Fridkin M. (1992) *Clin. Exp. Immunol.* **87**, 509.
2. Kelly SL, Adams SA, Robson SC, Kirsch RE, Shephard EG. (1994) *Biochem. J.* **298**, 689.
3. Adams SA, Kelly SL, Kirsch RE, Robson SC, Shephard EG. (1995) *Blood Coag. Fibrinol.* **6**, 693.
4. Shephard EG, Anderson RA, , Rosen O, Myer MS, Fridkin M, Strachan AF, De Beer FC. (1990) J. Immunol. **145**, 1469.
5. Kalvaria I, Rabinowitz S, Frith LOC, Kirsch RE. (1986) *Thrombosis Res.* **43**, 287.
6. Fraker PJ, Speck Jr. JC. (1978) *Biochem. Biophys. Res. Commun.* **80**, 849.
7. De Beer FC, Pepys MB. (1982) *J. Immunol. Methods.* **50**, 17.
8. Boyum A. (1968) *Scand. J. Lab. Invest.* **21** (Suppl. 97), 77.
9. Yassim R, Shefayk J, White JR, Tao W, Volpi M, Molsk TFP, Nacacche PH, Feinstein MB, Sharafi RI. (1985) *J. Cell Biol.* **101**, 182.
10. Brown EJ, Goodwin JL (1988) *J. Exp. Med.* **167**, 777.
11. Leytus SP, Kurachi K, Sakariassen KS, Davie EW. (1986) *Biochemistry.* **25**, 4855.
12. Leytus SP, Loeb KR, Hagen FS, Kurachi K, Davie EW. (1988) *Biochemistry.* **27**, 1067.
13. Laemmli UK. (1970) *Nature.* **227**, 680.
14. Towbin H, Staehelin T, Gordon T. (1979) *Proc. Natl. Acad. Sci. USA.* **76**, 4350.
15. Bangalore N, Travis J. (1994) *Biol. Chem. Hoppe Seyler.* **375**, 659.
16. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. (1995) *J. Cell Biol.* **131**, 775.
17. Pontremoli S, Melloni E, Michetti M, Sacco O, Sparatore B, Salamino F, Damiani G, Horecker BL. (1986) *Proc. Natl. Acad. Sci. USA.* **83**, 1685.
18. Owen CA, Campbell MA, Boukedes SS, Campbell EJ. (1997) *Amer. J. Physiol.* **272**, L385.
19. Gaudin P, Berthier S, Barro C, Zaoui P, Morel F. (1997) *Eur. J. Cell. Biol.* **72**, 345.

Chapter Five

Characterisation of the fibrinogen degrading activity of the neutrophil membrane

The findings presented in the previous chapter suggested the need to identify more carefully the nature of the membrane associated protease responsible for the degradation of fibrinogen, fibrin and CRP.[1-3] Recent reports of a membrane bound form of HNE[4-7] suggested that HNE might have been a constituent of neutrophil membrane preparations used in previous studies. This study sought to test the hypothesis that the proteolytic activity previously ascribed to a 600 kDa membrane associated protease was, at least in part, due to HNE.

Study design

- The conditioned medium of PMA (5-10ng/ml) stimulated neutrophils (due to the relative ease with which it is prepared) was used as the source of membrane associated protease for this study. Since the proteolytic activity of the conditioned medium has been reported to be identical to that of both neutrophil membrane and cytoskeleton fractions,[1-3] it was considered to be a representative source of the reported membrane associated protease.

- In this study the proteolytic activity of the conditioned medium was characterised with respect to its inhibitor profile, the nature of its proteolytic products as well as its molecular weight.

Methods

Chemicals and Reagents

The sources of chemicals and reagents used in this study are listed in *Appendix 1*, page 225.

Preparation & iodination of fibrinogen, neutrophil isolation, preparation of conditioned medium & solubilised cytoskeleton proteins

These procedures were performed as described previously (see chapter 4, *Methods*, page 107-108)

Preparation of granule enzymes

For the preparation of granule (lysosomal) enzymes, neutrophils were resuspended at 40 x 10^6 cells/ml and warmed to 37°C for 15 minutes. Cells were preincubated for 5 minutes with cytochalasin B (5μg/ml) and then stimulated with the chemotactic peptide N-formyl-L methionyl-leucyl-L-phenylalanine (FMLP) at a concentration of 1μM for a further 3 minutes. This is a very strong stimulus for the release of azurophil granules.[8] Following centrifugation at 2000 rpm for 3 minutes at 4°C, the cell free supernatant was collected and used immediately.

Degradation of [^{125}I]-labelled fibrinogen

[^{125}I]-labelled fibrinogen, in HBSS, was incubated with the various enzyme sources (conditioned medium of PMA stimulated neutrophils, granule enzyme preparations, purified HNE and purified cathepsin G) for various durations of time (5 minutes - 20 hours, 37°C), in a final reaction volume of 100μl. The concentrations of fibrinogen and the enzyme sources used in these experiments varied

and are clearly indicated in the results section. These concentrations were distinctly different from those used in previous studies[2,9] which investigated the degradation of fibrinogen at a physiological concentration of 2mg/ml by conditioned medium from 10 x 10^6 PMN. Changes to these concentrations allowed a detailed characterisation of the proteolytic activity of the conditioned medium. Degradation reactions were also performed in the presence of the specific HNE inhibitor, MeO-Suc-$(Ala)_2$-Pro-Val- CH_2Cl (1mM), and the cathepsin G inhibitors Z-Gly-Leu-Phe-CH_2Cl (0.1mM) and chymostatin (0.1mM). These inhibitors were preincubated with the enzyme source for 15 minutes at 37°C prior to the addition of fibrinogen. At selected time points, degradation was measured quantitatively as 10% TCA soluble peptide formation by the addition of 50% TCA (w/v) at 4°C to an aliquot of the reaction mixture to give a final concentration of 10% (w/v). For qualitative analysis, an aliquot of the reaction mixture was mixed with an equal volume of SDS-sample buffer (0.012 M Tris-HCl [pH 6.8] with 4% (w/v) SDS, and 24% (v/v) glycerol with or without 10% (v/v) 2-mercaptoethanol) and analysed by 5-20% SDS-PAGE (non-reduced) and 10% SDS-PAGE (reduced). Following electrophoresis, the gels were dried and the products visualised by autoradiography. Pre-stained molecular weight markers ranging in molecular weight from 14 to 200 kDa were used to identify apparent molecular mass values of fibrinogen degradation products.

Production of fibrinogen peptides and separation by h.p.l.c.

Unlabelled fibrinogen (2mg) was digested with HNE (2μg, 0.04 units), or neutrophil granule enzymes (from 10^6 PMN) at 37°C in a final volume of 2ml HBSS. The reaction was stopped at the specified times (see results) by the addition of 50% (w/v) TCA to a final volume of 10% (w/v). Following an overnight incubation at 4°C, the sample was centrifuged (2000 rpm, 10 minutes, 4°C) to remove precipitated protein. The supernatant was collected and the TCA was extracted four times with 8ml of diethyl ether. The remaining peptide mixture was then lyophilised and resuspended in deionised H_2O at 10% the original volume. The peptides were separated using a Merck Hibar Column (250 x 4mm) prepacked with LiChrosorb RP-18 (5μM). H.p.l.c. was performed using a Waters Associates liquid

chromatograph with a 600E controller, solvent delivery system (model 6000), automatic injector (model 710B), variable wavelength detector (model 484 set at 220nm), and a flat bed recorder. The column was loaded with peptide aliquots (volumes ranging up to 120µl) and eluted using a binary gradient formed from 0.1% trifluoroacetic acid (solution A) and 0.1% trifluoroacetic acid in acetonitrile/water (75:25, solution B). The initial condition of 95% solution A and 5% solution B was run isocratically for 7 minutes and then followed by a linear gradient for a further 53 minutes to 30% of solution A and 70% of solution B at a flow rate of 1ml/min.

Zymography

The method used for polyacrylamide gel electrophoresis in SDS containing gels was essentially that described by Laemmli[10] except that the protein substrates (casein, fibrinogen and CRP) were copolymerised within the polyacrylamide gels.[11] The various substrates were incorporated into SDS polyacrylamide gels (3-13%) at final concentrations ranging from 50µg to 1mg/ml. Samples were mixed with an equal volume of SDS-sample buffer (see above) and allowed to stand at room temperature for 30 minutes before being applied to the gels. Electrophoresis was performed at a constant voltage (150V) at 4°C, after which the gels were washed to remove SDS. This involved successive washes with 50ml portions of 2.5% (w/v) Triton X-100 in distilled water (two washes, 10 minutes each) and 2.5% (w/v) Triton X-100 in Tris-HCl (50mM), pH 7.4 (two washes, 10 minutes each). The excess triton was removed by 2 washes (10 minutes each) in PBS (100ml). Following washing steps, the gels were placed in a dish with 100ml of PBS and incubated at 37°C for 24 hours. Gels were then stained with coomasie blue (methanol 40% [v/v], acetic acid 16 % [v/v], coomassie brilliant blue 0.12% [w/v]) and destained to reveal zones of substrate lysis.

Results

1. The effect of the specific HNE inhibitor, MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl.

Neutrophil conditioned medium

The specific HNE inhibitor, MeO-Suc-$(Ala)_2$-Pro-Val- CH_2Cl, was found to cause marked inhibition of the fibrinogen degrading activity of the conditioned medium, as measured by TCA-soluble peptide formation and the integrity of fibrinogen on SDS-PAGE (figures 1 and 2). This inhibitory effect was particularly evident at earlier time points but gradually diminished with the progression of time. (figures 1a and b).

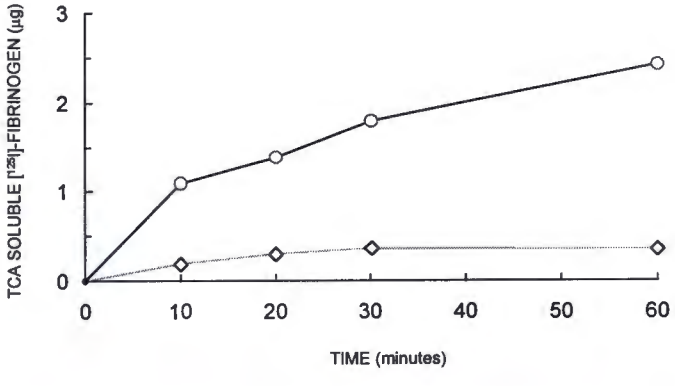


Figure 1a: The effect of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl on the [^{125}I]-labelled-fibrinogen degrading activity of the conditioned medium. *[^{125}I]-labelled fibrinogen (10µg) was incubated (37°C, 0-60 minutes) with the conditioned medium from 10^6 PMA (5ng/ml) stimulated neutrophils in a final volume of 100µl. Reactions were performed in the absence (-O-) and presence (-◊-) of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl (1mM). Degradation was measured as 10% (w/v) TCA soluble peptide formation. Each data point represents a single measurement.*

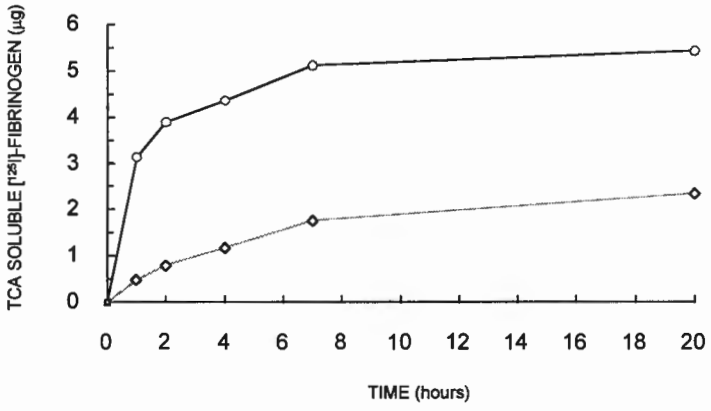


Figure 1b: The inhibitory effect of MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl (1mM) at later time points. *The [^{125}I]-labelled fibrinogen degrading activity of the conditioned medium of PMA stimulated neutrophils, in the absence (-O-) and presence (-◊-) of MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl, was tested at later time points (1-24 hours) under the same conditions as described in figure1a. Each data point represents a single measurement. An additional experiment gave similar results (see page Appendix 3, page 231)*

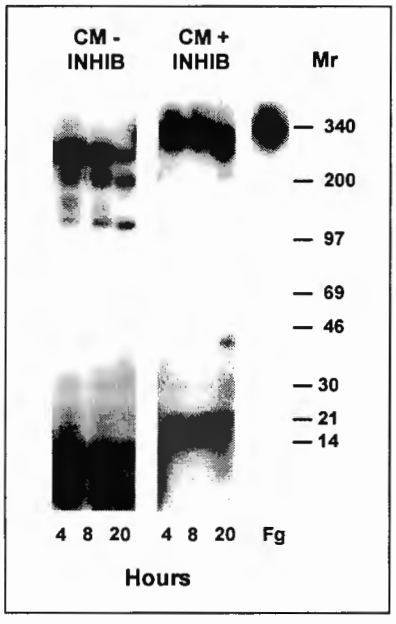


Figure 2: Degradation of [^{125}I]-fibrinogen by the neutrophil conditioned medium in the presence and absence of a specific inhibitor of HNE (MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl). *[^{125}I]-labelled fibrinogen (10µg) was incubated (37°C, 4-20 hours) with the conditioned medium (CM) from 10^6 PMA (5ng/ml) stimulated neutrophils in a final volume of 100µl. Reactions were also performed in the presence of MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl (1mM). At selected time points, the nature of the degradation products was assessed by SDS-PAGE (5-20%, non-reduced). The left hand panel shows the fibrinogen degradation products generated by the conditioned medium at 4, 8, and 20 hours, whilst the right hand panel shows the fibrinogen degradation products generated by the conditioned medium in the presence of MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl at the corresponding time points. On the far right is non degraded fibrinogen.*

Intact neutrophils

MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl was also found to inhibit the fibrinogenolytic activity of intact neutrophils. The specific HNE inhibitor was found to inhibit neutrophil mediated generation of TCA soluble [^{125}I]-labelled fibrinogen peptides by 83%, 58%, 57% and 50% at 10 minutes, 30 minutes, 1 hour and 5 hours respectively. SDS-PAGE analysis of these samples confirmed the inhibitory effect of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl on fibrinogenolysis (Figure 3).

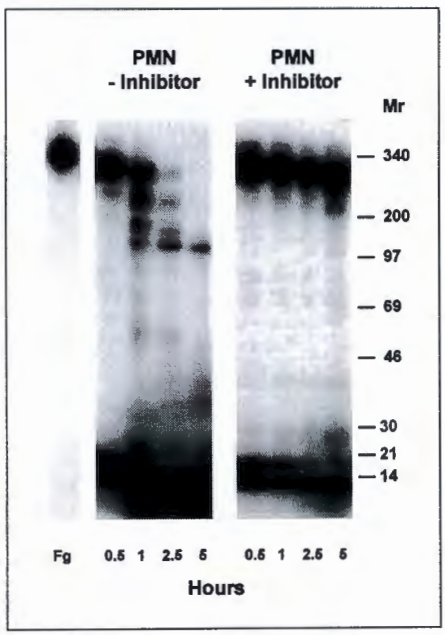


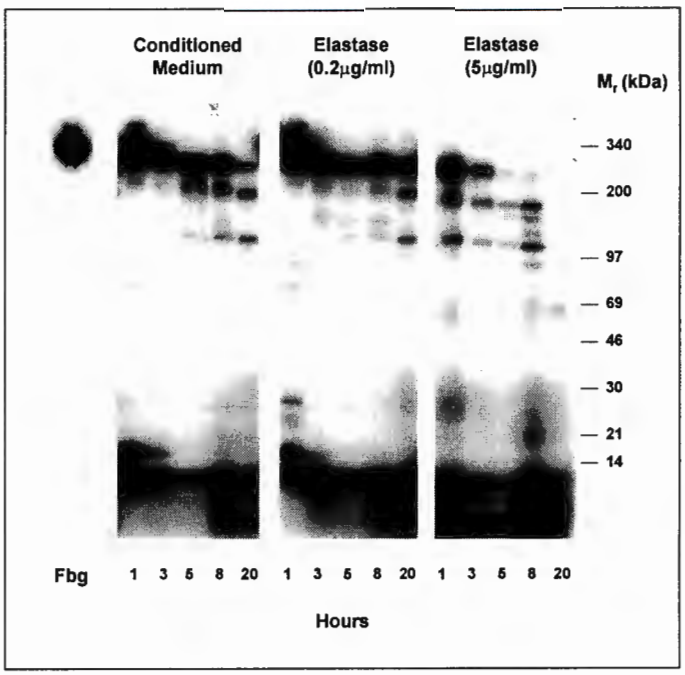
Figure 3: The effect of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl on the degradation of [^{125}I]-fibrinogen by PMA stimulated neutrophils. *[^{125}I]-labelled fibrinogen (25μg) was incubated (37°C, 30 minutes - 5 hours) with 2.5 x 10^5 PMA (5ng/ml) stimulated neutrophils in a final volume of 50μl. Prior to the addition of [^{125}I]-labelled fibrinogen, neutrophils were incubated (37°C, 15 minutes) in the presence or absence of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl (1mM). At selected time points, samples were subjected to SDS-PAGE (non-reduced, 5-20% polyacrylamide) and autoradiography. The left lane shows non-degraded fibrinogen. The left hand panel shows fibrinogen degradation products generated at 0.5, 1, 2.5 and 5 hr by PMA stimulated neutrophils. The right hand panel shows fibrinogen degradation products generated by PMA stimulated neutrophils in the presence of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl at the corresponding time points.*

2. Comparison of the fibrinogen degradation products generated by the conditioned medium to those generated by HNE

SDS-PAGE and autoradiography

The [^{125}I]-labelled fibrinogen degradation products generated by the conditioned medium were compared to those generated by HNE. HNE, 5μg/ml, generated fibrinogen degradation products at 20 hours with apparent molecular weight values distinctly different from those generated by the conditioned medium. However, when the concentration was reduced to 0.2μg/ml, HNE generated fibrinogen degradation products with very similar apparent molecular mass values to those produced by the neutrophil conditioned medium (figures 4a and b).

4(a)



(b)

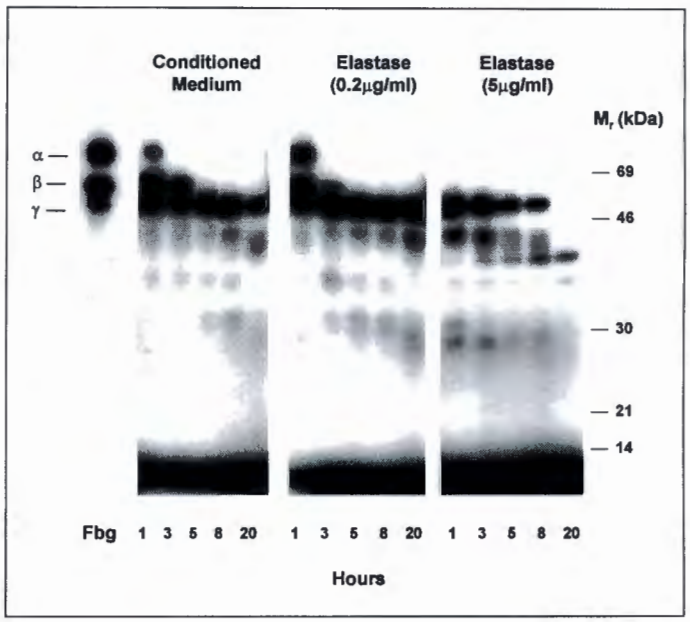


Figure 4: Degradation of [^{125}I]-labelled fibrinogen by the conditioned medium of PMA stimulated neutrophils compared to that by human neutrophil elastase. *[^{125}I]-labelled fibrinogen (20μg) was incubated (37°C, 1-20 hours) with the conditioned medium from 10^6 PMA stimulated neutrophils in a final volume of 100μl. At selected time points the nature of the products generated was assessed by SDS-PAGE under both reducing and non-reducing conditions. (a) Non-reducing, 5-20% polyacrylamide gel: The left hand lane contains non-degraded fibrinogen. The next panel shows the fibrinogen degradation products generated by the conditioned medium at 1,3,5,8 and 20 hours respectively. The next two panels show the fibrinogen degradation products generated by low dose (0.2μg/ml) and high dose (5μg/ml) HNE at the corresponding time points. (b) Reducing, 10% polyacrylamide gel: The fibrinogen degradation products generated by the conditioned medium and HNE (as described above) were also analysed under reducing conditions.*

High performance liquid chromatography

High performance liquid chromatography (h.p.l.c.) was used to compare the pattern of fibrinogen peptides generated by low dose HNE to that previously reported for the conditioned medium. As a baseline against which to compare HNE generated peptides, an aliquot of TCA soluble fibrinogen peptides generated by the neutrophil conditioned medium was obtained (with permission) from

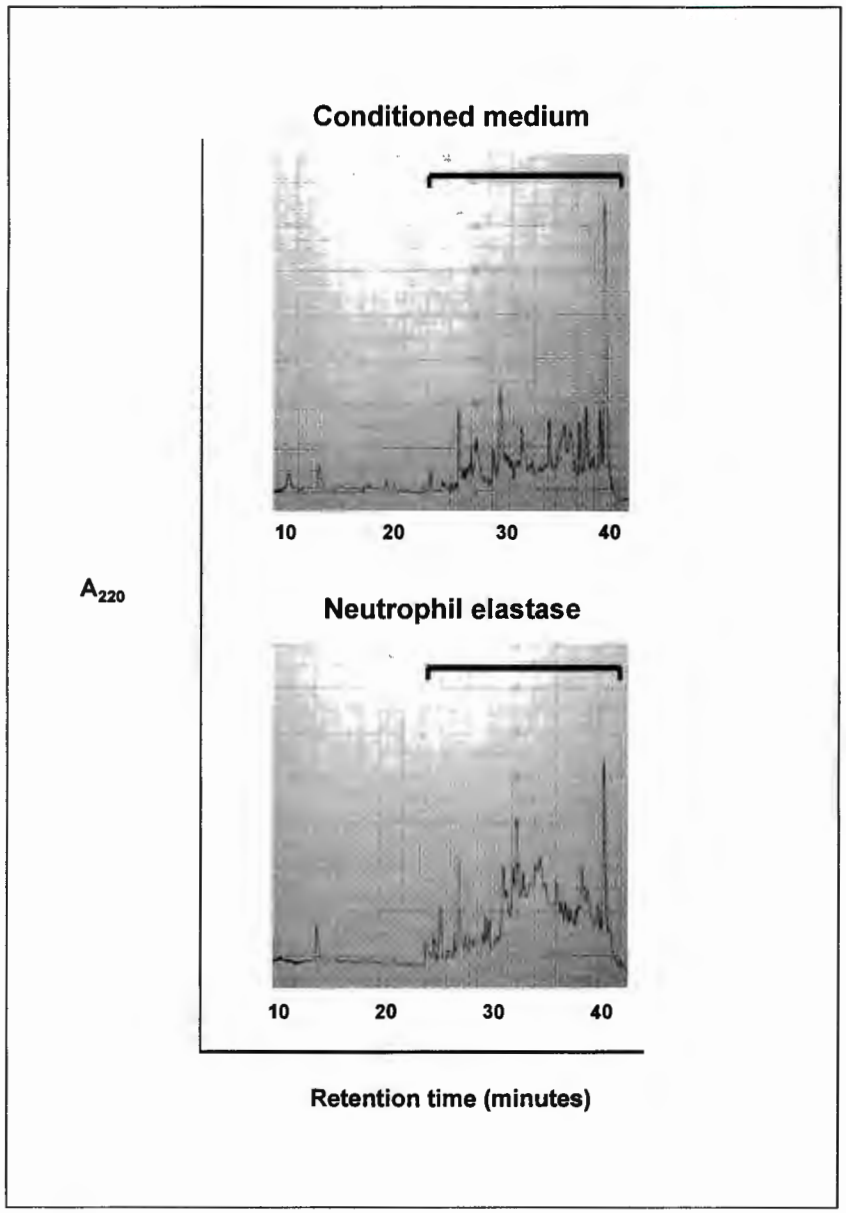


Figure 5: H.p.l.c. separation of (10%) trichloroacetic acid soluble fibrnogen peptides. *Unlabelled fibrinogen (2mg/ml) was incubated (37°C, 9 hr) with conditioned medium from PMA stimulated neutrophils (10 x 10^6) or purified HNE (2μg/ml) in a final volume of 2ml. TCA soluble peptides obtained were separated using a C18 reverse phase column (flow rate 1ml/min) as described in the Methods section). This figure shows the h.p.l.c. profiles of fibrinogen peptides generated by the neutrophil conditioned medium (top) and by purified HNE (bottom) at 9 hours.*

a stored batch used in a previous study.* (The ratio of HNE to fibrinogen (2μg:2mg) was the same as that which generated fibrinogen degradation products shown in the central panels of figures 4a and b). The peptide profile generated by HNE appeared markedly different to that which had been published previously,[2] and bore a much closer resemblance to the peptide profile generated by the neutrophil conditioned medium, with the majority of the peptides having a retention time of between 25-40 minutes (see brackets, figure 5). The HNE in the previous study was from Sigma (specific activity 60 units/ml) unlike the HNE used this study which was from Calbiochem (specific activity 20 units/ml). Thus depending on the activity of the HNE, vastly differing peptide profiles could be obtained.

3. The contribution of cathepsin G to the proteolytic activity of the conditioned medium.

The persistence of fibrinogen degradation in the presence of the specific HNE inhibitor, MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl (Figure 1), suggested the presence of a second protease in the conditioned medium. To determine whether this residual activity was due to another azurophil granule protease, cathepsin G, further inhibitor studies were performed. Two inhibitors of cathepsin G, namely the choromethyl ketone inhibitor, Z-Gly-Leu-Phe-CMK (0.1mM) and the reversible chymotrypsin inhibitor, chymostatin (0.1mM), were tested for their ability to inhibit the fibrinogen degrading activity of the conditioned medium. These inhibitors were used alone or in combination with the specific HNE inhibitor MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl (1mM).

The two inhibitors of cathepsin G were found to have a marked inhibitory effect on the residual (MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl resistant) fibrinogenolytic activity of the neutrophil conditioned medium. When used in combination, MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl and Z-Gly-Leu-Phe-CMK were found to inhibit the

* This was obtained from stores of Sharon Kelly (Liver Research Centre, UCT, SA). These TCA soluble peptides had been generated during in an earlier study[2] by digesting fibrinogen (10mg/ml) with the conditioned medium from 10 x 10^6 neutrophils at 37°C for 9 hours. Aliquots of peptides were stored at -70°C.

fibrinogenolytic activity of the neutrophil conditioned medium by 100 % at 2 hours, 95% at 4 hours, 93% at 7 hours and 86% at 20 hours (Figure 6a).

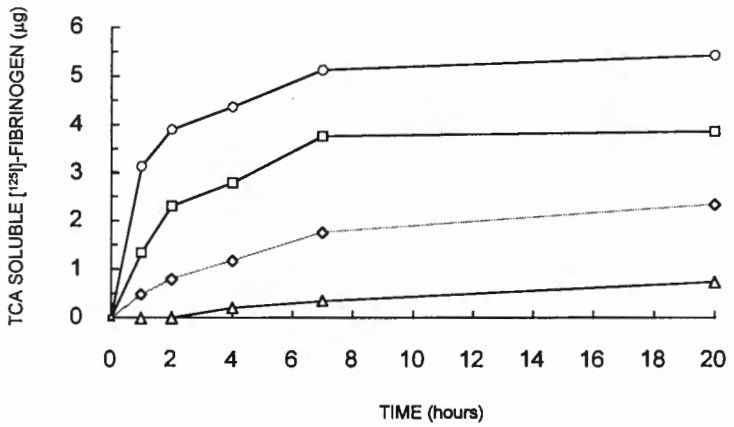


Figure 6a: The effect the specific cathepsin G inhibitor (Z-Gly-Leu-Phe-CMK) on the [^{125}I]-labelled fibrinogen degrading activity of the conditioned medium. *[^{125}I]-labelled fibrinogen (10µg) was incubated (37°C,1-20 hours) with conditioned medium (from 10^6 PMA stimulated neutrophils) in a final volume of 100µl. Reactions were performed in the presence and absence of Z-Gly-Leu-Phe-CMK (0.1mM), used alone or in combination with MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl (1mM). At selected time points, 10% (w/v) TCA soluble [^{125}I]-labelled fibrinogen peptide formation was measured in each group as described earlier. The four groups are represented as follows: No inhibitor (-O-); Z-Gly-Leu-Phe-CMK alone (-□-); MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl alone (-◊-); Z-Gly-Leu-Phe-CMK plus MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl (-Δ-). Each data point represents a single measurement.*

Similarly, when chymostatin was used in combination with the specific HNE inhibitor, the fibrinogenolytic activity of the conditioned medium was reduced by 93%, 94% and 93% at 1 hour, 2 hours and 3 hours respectively. At 5 hours there was 87% inhibition and by 20 hours it decreased to 83% (figure 6b).

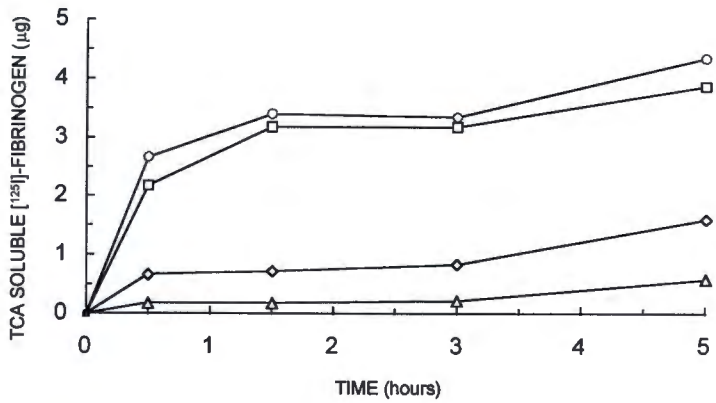


Figure 6b. The effect of chymostatin on the [^{125}I]-labelled fibrinogen degrading activity of the conditioned medium. *[^{125}I]-fibrinogen (10µg) was incubated (37°C, 0.5-5 hours) with conditioned medium (from 10^6 PMA stimulated neutrophils) in a final volume of 100µl. Reactions were performed in the presence and absence of chymostatin (0.1mM), used alone or in combination with MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl (1mM). At selected time points, 10% (w/v) TCA soluble [^{125}I]-labelled fibrinogen peptide formation was measured in each group. The four groups are represented as follows: No inhibitor (-○-),chymostatin alone (-□-); MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl alone (-◊-);chymostatin plus MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl (-Δ-). Each data point represents a single measurement.*

Fibrinogen degradation products generated by the conditioned medium in the presence of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl were compared to those generated by cathepsin G. The pattern of fibrinogen degradation by the two proteases appeared almost identical (Figure 7a and 7b), with complete degradation of the Aα chain occurring before significant digestion of Bβ or γ-chains (Figure 7b, next page).

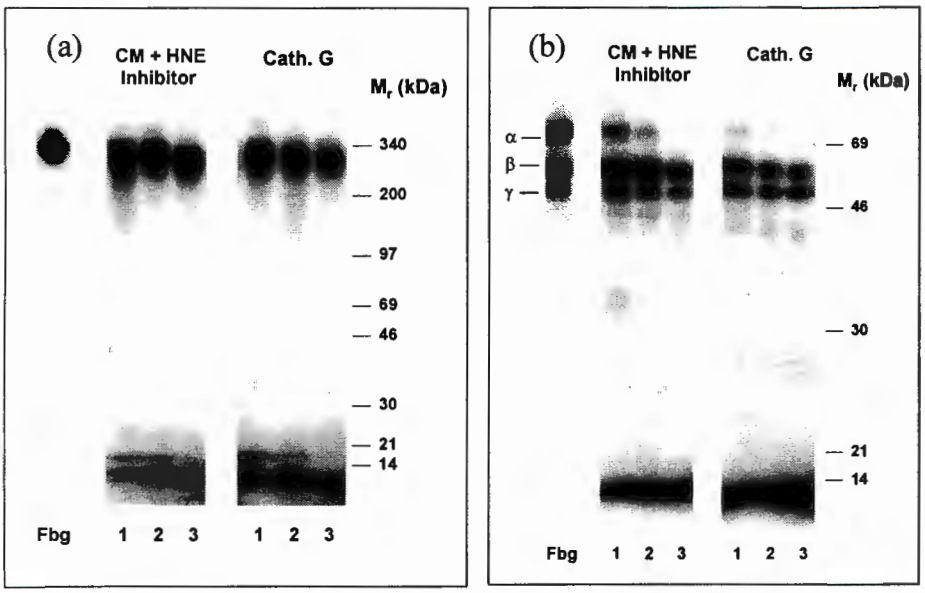


Figure 7. Proteolysis of [^{125}I]-labelled fibrinogen by MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl-inhibited conditioned medium compared to that by cathepsin G. *Conditioned medium (CM) from 10^6 PMA stimulated neutrophils, was incubated in the presence of MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl (1mM), with [^{125}I]-labelled fibrinogen (1μg) for various durations of time. In parallel experiments, purified cathepsin G (0.025μg) was incubated [^{125}I]-labelled fibrinogen (1μg). Samples were then subjected to SDS-PAGE and autoradiography. (a) Non-reducing gel, 5-20% polyacrylamide. The left lane shows non-degraded fibrinogen. In the left hand panel lanes 1-3 represent fibrinogen degradation products generated by the MeO-Suc-(Ala)$_2$-Pro-Val-CH_2Cl-inhibited conditioned medium at 2.5, 5 and 24 hours respectively. In the right hand panel, lanes 1-3 represent the fibrinogen degradation products generated by cathepsin G at 1, 2.5 and 5 hours respectively. (b) The same products were also analysed under reducing conditions in a 10% polyacrylamide gel.*

4. Comparison of the fibrinogen degradation products generated by the conditioned medium to those generated by granule proteases.

Data acquired thus far suggested that the proteolytic activity of the conditioned medium was due to a combination of the azurophil granule proteases HNE and cathepsin G. To further substantiate this suggestion, the [^{125}I]-labelled fibrinogen degradation products generated by the conditioned medium were compared to those generated by preparations rich in azurophil granule proteases (see

Methods). When used at low concentrations, granule protease preparations generated fibrinogen degradation products with apparent molecular mass values which closely resembled those of products generated by neutrophil conditioned medium. (see figure 8).

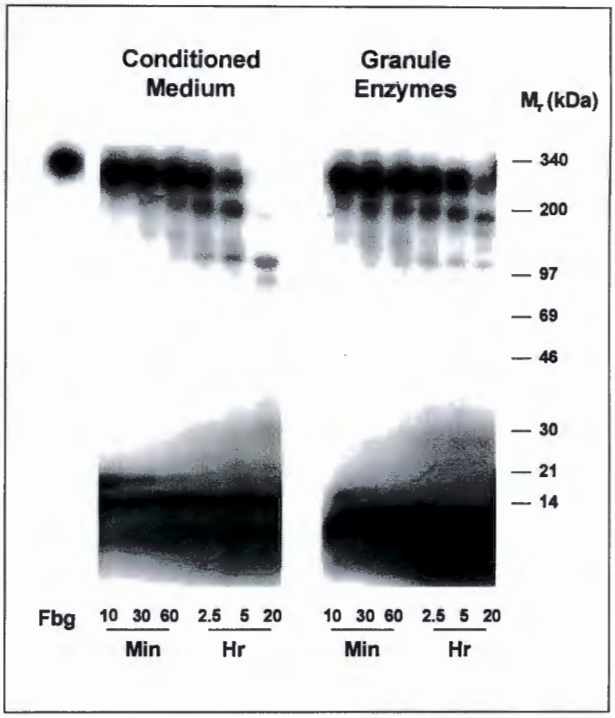


Figure 8: Degradation of [^{125}I]-labelled fibrinogen: Conditioned medium versus granule enzymes. *[^{125}I]-labelled fibrinogen (2.5μg) was incubated (37°C, 10 minutes -20 hours) with either conditioned medium (from 10^6 neutrophils) or with granule enzymes (released from 10^5 neutrophils) in a final volume of 100μl. At selected time points samples were subjected to SDS-PAGE (5-20%, non-reducing) and autoradiography. The fibrinogen degradation products generated by the conditioned medium (left hand panel) and by granule enzymes (right hand panel) are indicated above. The fibrinogen standard is in the extreme left hand lane.*

The h.p.l.c. profiles of fibrinogen peptides generated by diluted granule preparations appeared similar to those generated by the neutrophil conditioned medium, most peptides having retention times of between 25-40 minutes (Figure 9).This profile was very different to that published previously for fibrinogen peptides generated by granule enzymes.

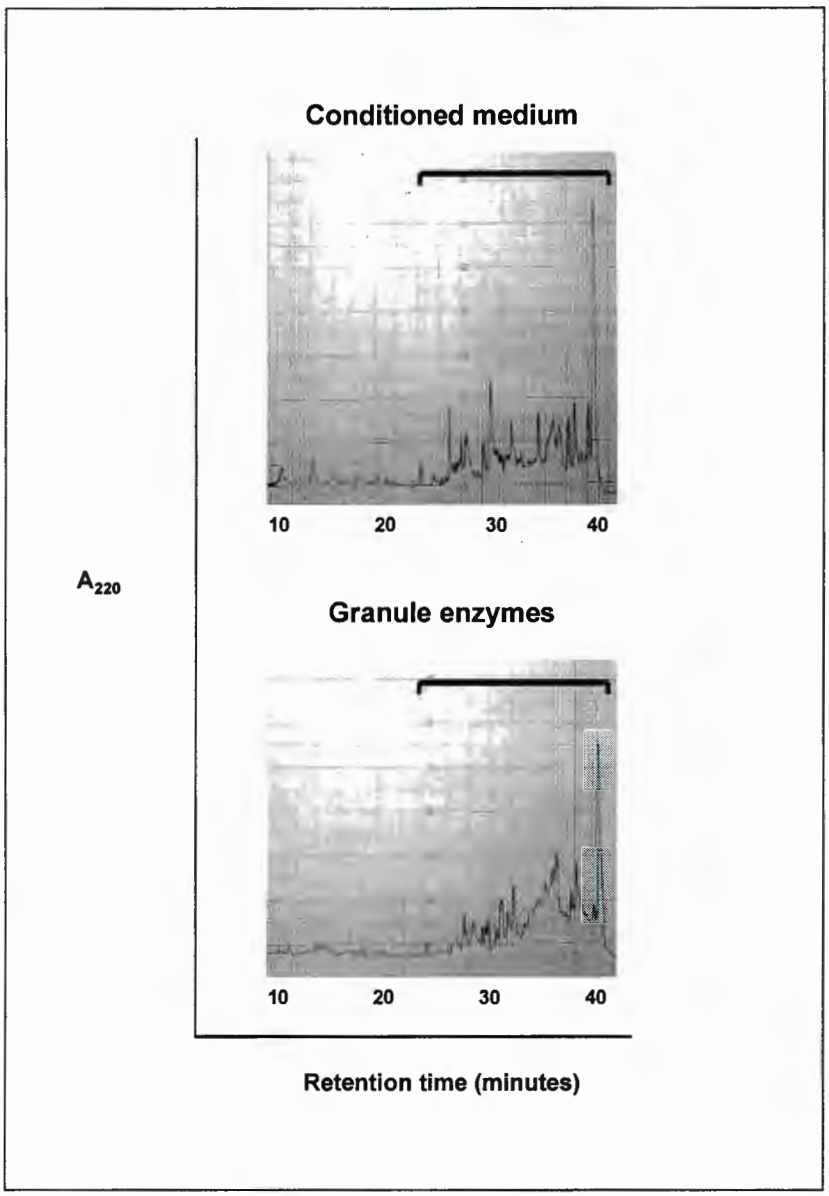


Figure 9: H.p.l.c. separation of (10%) trichloroacetic acid soluble fibrinogen peptides. *Unlabelled fibrinogen (2mg/ml) was incubated (37°C, 9 hr) with conditioned medium (from 10 x 10^6 PMA stimulated neutrophils)* or granule enzymes (from 10^6neutrophils) in a final volume of 2ml. TCA soluble peptides obtained were separated using a C18 reverse phase column (flow rate 1ml/min) as described in the Methods section. This figure shows the h.p.l.c. profiles of fibrinogen peptides generated by neutrophil conditioned medium (top) and granule enzymes (bottom) at 9 hours. (* see footnote page 137)*

5. Identification of the apparent molecular mass of the membrane associated proteolytic activity

To determine the molecular mass of the proteolytic activity associated with the conditioned medium, a highly sensitive protease detection technique, known as zymography,[11] was used (see *Methods*). Due to availability at the time, the proteolytic activity of neutrophil cytoskeleton preparations was analysed initially.

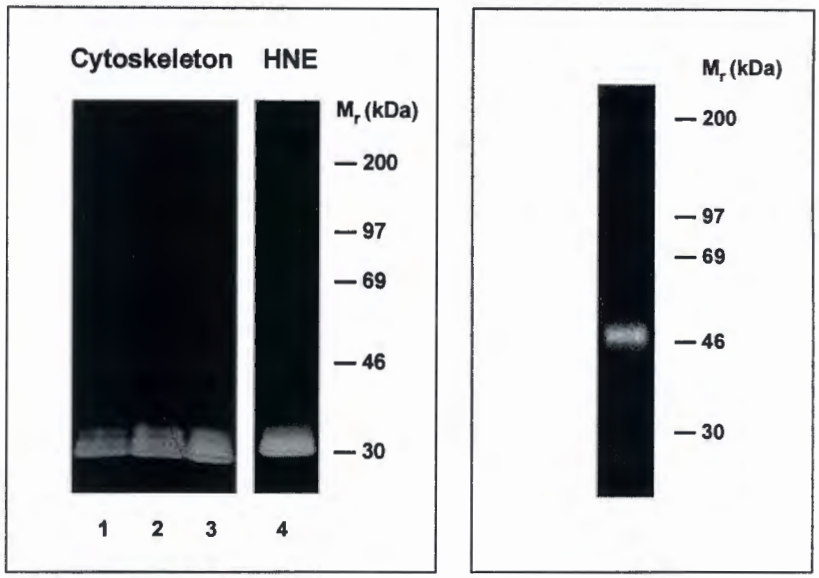


Figure 10: Zymography of cytoskeleton preparations.
(a) Casein (1mg/ml) was copolymerised within a 3-13% polyacrylamide gel. The neutrophil cytoskeleton fraction (from 2.5-, 5- and 10 x 10^6 neutrophils) was loaded in wells 1-3 respectively. Purified HNE (0.5μg) was loaded in well 4. (Samples were boiled in SDS sample buffer prior to electrophoresis). Following removal of SDS, gels were incubated at 37°C overnight prior to staining with coomassie blue.
(b) Fibrinogen (1mg/ml) was copolymerised as above. Neutrophil cytoskeleton prepared from 20 x 10^6 cells was subjected to zymography as described above.

Casein, widely regarded as a universal substrate which is degraded by almost all proteases,[12] was used as the substrate in initial experiments (Figure 10a). The caseinolytic activity of soluble cytoskeleton proteins was located in 30 kDa region of the gel and appeared to be comprised of three separate bands. Significantly no

proteolytic activity was detected in regions of the gel previously reported to contain the proteolytic activity of the 600 kDa protease. Purified HNE appeared to migrate to the same position as the proteolytic activity of the cytoskeleton preparation (Figure 10a, track 4).

When cytoskeleton preparations were subjected to zymography in *fibrinogen* containing gels (Figure 10b), the fibrinogen degrading activity migrated to a discrete band in the 50 kDa region of the gel (Figure 10b). This most likely reflects altered mobility of proteolytic activity in fibrinogen- compared to casein-containing zymograms (see discussion).

In these experiments, samples were boiled prior to electrophoresis, a step which is generally omitted due to the risk of irreversible denaturation and loss of catalytic activity.[11] As it was possible that heat-sensitive proteolytic activity might have been lost as a result of the boiling step, experiments were repeated using unboiled samples. A band in the 69-97 kDa region of the gel appeared when *unboiled* cytoskeleton preparations were subjected to casein zymography (figure 11, left hand panel, track 1). Interestingly, a band of identical electrophoretic mobility was observed in zymograms of unboiled HNE (figure 11, left panel, right track). The similar zymographic characteristics of cytoskeleton preparations and HNE suggested that HNE was the dominant protease in cytoskeleton preparations. The additional band which appeared in zymograms of non-boiled samples was most likely due to aggregate formation (rather than a distinct protease) as it was also present in preparations of purified HNE.

The caseinolytic activity of both cytoskeleton preparations and HNE was completely abolished if samples were pre-incubated with MeO-Suc-$(Ala)_2$-Pro-Val- CH_2Cl (1mM) prior to electrophoresis (figure 11, right hand panel), further suggesting that the proteolytic activity of cytoskeleton preparations was due to HNE.

The proteolytic activity associated with the neutrophil *conditioned medium* was subsequently tested in both fibrinogen- and CRP-zymograms. Fibrinogen zymography revealed a zone of clearing corresponding to an apparent molecular mass of 30-60 kDa, and no

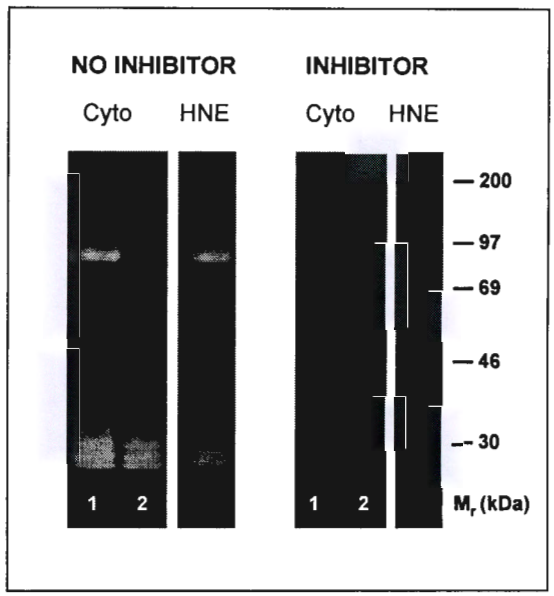
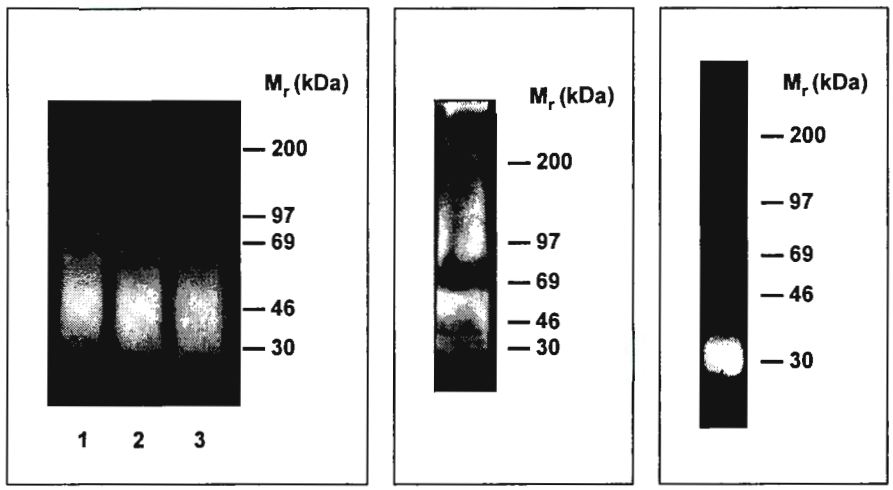


Figure 11: The effect of MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl on the caseinolytic activity of the neutrophil cytoskeleton and HNE. *The left hand panel shows casein gels (as in figure 10a) loaded with neutrophil cytoskeleton (Cyto) from 5 x 10^6 neutrophils (non-boiled or boiled - lanes 1 and 2 respectively) or purified HNE (non-boiled). The right hand panel shows identical samples which had been pre-incubated with MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl (37°C, 15 minutes) prior to electrophoresis.*

zones of clearing in the high molecular weight regions of the gel (Figure 12a). The same pattern was observed in 3 consecutive zymograms. In one ultraconcentrated preparation (in which a large amount of insoluble material was present) proteolysis extended beyond 69 kDa and a discrete zone of proteolysis was observed at the very top of the gel (Figure 12b). At lower concentrations this proteolytic zone was never observed, suggesting that it was most likely the result of aggregate formation which accompanies concentrating steps. CRP zymograms consistently revealed a zone of clearing confined to the 30 kDa region of the gel (Figure 12c).



(a) (b) (c)

Figure 12: Zymography of the conditioned medium.
(a) Fibrinogen (0.08mg/ml) was co-polymerised within a (3-13%) polyacrylamide gel. Neutrophil conditioned medium from 1.2 x 10^6, 6 x 10^5 and 3 x 10^5 PMA stimulated neutrophils was loaded in tracks 1, 2, and 3 respectively. Following the removal of SDS, gels were incubated for 36 hours at 37°C prior to staining with coomasie blue.
(b) Fibrinogen (0.1mg/ml) zymography (as above) of conditioned medium from 20 x 10^6 PMA stimulated neutrophils, lyophilised and reconstituted in dH_2O at 1/50 original volume.
(c) C-reactive protein (CRP) (0.1mg/ml) zymography of conditioned medium from 1.2 x 10^5 PMA stimulated neutrophils.

Discussion

This study sought to identify more carefully the nature of the proteolytic activity previously ascribed to a 600 kDa membrane associated protease. On the basis of its inhibitor profile, its fibrinogen degradation products, and its apparent molecular mass, this study suggests that the proteolytic activity of the neutrophil membrane is due to proteases of the azurophil granule. In the discussion which follows, data obtained during this study is reviewed in the context of earlier studies. Thereafter, the mechanisms by which these proteases become associated with the three neutrophil fractions are considered.

The effect of the specific HNE inhibitor, MeO-Suc-$(Ala)_2$-Pro-Val- CH_2Cl

Several observations made during attempts to purify the 600 kDa protease suggested that HNE might contaminate neutrophil fractions reported to contain this protease. The presence of HNE was initially investigated by means of a widely used specific inhibitor of HNE, MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl.[6,7,13,14] The conditioned medium of PMA stimulated neutrophils was used as the source of this protease in this study for two reasons. Firstly, it is prepared more easily than the other two neutrophil fractions, and secondly, earlier studies in which the 600 kDa protease was characterised, used the conditioned medium as the principal source of this protease.

The fibrinogen degrading activity of the conditioned medium as well as that of intact neutrophils was found to be very sensitive to MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl, particularly at earlier time points. There was a gradual decrease in its inhibitory effect on the fibrinogenolytic activity of the conditioned medium (and intact cells) over time. At 20 hours, inhibition by MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl of the fibrinogenolytic activity of the conditioned medium was close to the previously reported 50%.[2]

The conditioned medium has also been reported to be resistant to other two specific HNE inhibitors, Suc-$(Ala)_3$-CH_2Cl and Suc-$(Ala)_2$-Val-CH_2Cl.[2] However, subsequent investigation has revealed that the nitroanilide (NA) derivatives of the abovementioned peptides (i.e. Suc-$(Ala)_3$-NA and Suc-$(Ala)_2$-Val-NA) rather than the chloromethyl ketone derivatives were used. While peptide chloromethyl ketones are often used as irreversible inhibitors, peptide nitroanilides function as chromogenic substrates. The chloromethyl ketone group alkylates the active site histidine forming a tetrahedral adduct with the active site serine. This causes inactivation of the protease[15] (Figure 13).

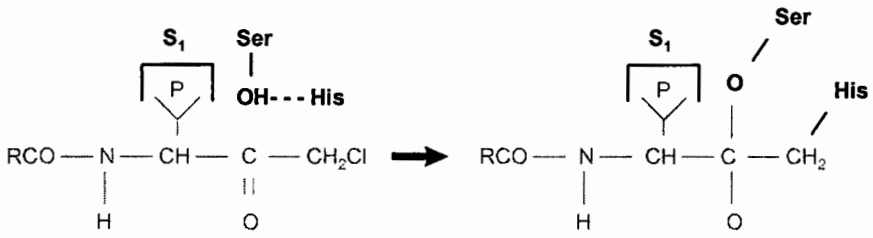


Figure 13. Serine protease inactivation by peptide chloromethyl ketone inhibitors. *The peptide chloromethyl ketone inhibitor (grey) binds to the serine protease substrate binding site (S_1) via a targeting peptide sequence (P). This is followed by the alkylation of the active site histidine to form a tetrahedral adduct with the active site serine. This results in irreversible inactivation of the protease. (Adapted from Reference 15)*

The chloromethyl ketone group therefore plays a central role in the inhibitory activity of these peptide inhibitors. Nitroanilide derivatives, on the other hand, do not alter the active site, and at most, may competitively inhibit protease activity. However, as their efficacy as HNE inhibitors had not established prior to their use on the conditioned medium, their reported lack of inhibitory activity with respect to the conditioned medium is impossible to interpret. Whilst a review of recent studies indicates that MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl is the most widely used inhibitor of HNE,[6,7,13-15] no reports regarding the use of the two abovementioned NA-peptide substrates as inhibitors of HNE have been found.

It was thus clear that inhibitor profiles could no longer reliably exclude the presence of HNE in the conditioned medium.

Comparison of the fibrinogen degradation products generated by conditioned medium and HNE

Although it had been reported that the neutrophil conditioned medium generated fibrinogen degradation products with distinctly different apparent molecular mass values from those produced by HNE,[2] data obtained during inhibitor studies suggested that this should be re-investigated. A comparison of the proteolytic activity of HNE and conditioned medium was therefore made over a wide range of HNE concentrations. Consistent with earlier reports,[2] it was found that when used at relatively high concentrations, the fibrinogen degradation products generated by HNE appeared distinctly different to those generated by the conditioned medium. However, if used at sufficiently low concentrations, HNE generated fibrinogen degradation products which appeared very similar to those produced by the conditioned medium of PMA stimulated neutrophils.
When HNE was used at 0.04 units/2mg fibrinogen (as opposed to 0.12 units/2mg fibrinogen used in previous studies),[2] peptide profiles bore a much closer resemblance to those generated by the conditioned medium, and were vastly different to those previously reported for HNE.[2] This previous study had shown that the peptides generated by the conditioned medium of PMA stimulated neutrophils still possessed HNE sensitive sites.[2] Thus the process and extent of fibrinogenolysis by HNE appears to be dependent on protease concentration.

These findings suggest that the previously reported differences in the pattern of fibrin(ogen) degradation between the conditioned medium and HNE, most likely represented concentration effects rather than differences in proteolytic activity. Taken together with the inhibition data, this strongly suggests the presence of HNE in the conditioned medium of PMA stimulated neutrophils.

The fibrinogen peptide profiles generated by HNE and conditioned medium were not identical, however, and differences were more than likely due to the presence of one or more additional proteases in the

conditioned medium. Two other factors suggested that the conditioned medium contained additional proteases besides HNE. Firstly, as the release of HNE is not selective, but accompanied by the release of other azurophil granule proteases, its presence in the conditioned medium would imply the presence of other azurophil granule proteases as well. Secondly, consistent with previous reports,[2] the present study found the conditioned medium to possess residual fibrinogen degrading activity which persisted in the presence of the specific HNE inhibitor, MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl.

The contribution of cathepsin G to the proteolytic activity of the conditioned medium.

It was postulated that the "MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl resistant" proteolytic activity of the conditioned medium was largely attributable to a second major azurophil granule serine protease, cathepsin G. Inhibitor studies revealed that this residual activity was sensitive to two inhibitors of cathepsin G, namely the specific chloromethyl ketone cathepsin G inhibitor, Z-Gly-Leu-Phe-CMK, as well as the chymotrypsin inhibitor, chymostatin. When Z-Gly-Leu-Phe-CMK was used in combination with MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl, the fibrinogenolytic activity of the neutrophil conditioned medium was inhibited by nearly 100% up to 3 hours. Chymostatin, in combination with MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl, inhibited the fibrinogen degrading activity by over 90% over the corresponding time period. At later time points there was a gradual decrease in the levels of inhibition until, at 20 hours, inhibition levels were 86% and 83% for Z-Gly-Leu-Phe-CMK and chymostatin (used in combination with MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl) respectively. Analysis of the fibrinogen degradation products generated by the conditioned medium in the presence of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl, revealed them to be very similar to those produced by purified cathepsin G. Together this data suggested that cathepsin G was responsible for the bulk of the "non-HNE" proteolytic activity of the conditioned medium.

Whilst previous studies had reported the fibrinogen degrading activity of the conditioned medium to be resistant to a specific chloromethyl ketone inhibitor of cathepsin G,[2] further investigation

revealed that, once again, a nitroanilide rather than chloromethyl ketone derivative of this peptide substrate had been used.

A small amount of fibrinogen degrading activity (mainly evident at later time points) was noted to persist in the presence of HNE and cathepsin G inhibitors. It is likely that this residual fibrinogen degrading activity was due the third serine protease of the azurophil granule, proteinase 3 (PR3). Like HNE and cathepsin G, this protease has also been shown to be associated with the neutrophil membrane.[16] Whilst the involvement of PR3 was not investigated in this study, previous reports that the proteolytic activity of the conditioned medium is completely abolished by the serine protease inhibitor PMSF, suggests that this residual activity is due to a serine protease.[2] Furthermore, the presence of HNE and cathepsin G in the conditioned medium implies the presence of other azurophil granule constituents which would include PR3.

It is interesting to note that a 21 amino acid peptide, cleaved from the N-terminus of the Aα chain of fibrinogen, has previously been identified in the peptide mixture generated by the conditioned medium of PMA stimulated neutrophils in the presence of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl.[2] This peptide was once considered to be produced exclusively by HNE and its presence in plasma had been used as an indicator of α_1-antitrypsin* deficiency.[17,18] From this study it appears that cathepsin G (and/or perhaps PR3) may also generate this peptide and account for the of MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl-insensitive pathway for producing this peptide noted previously.[2]

Comparison of the fibrinogen degradation products generated by conditioned medium and granule proteases.

Data accumulated thus far suggested that the proteolytic activity of the neutrophil conditioned medium was due to azurophil granule proteases. Although it had been reported that neutrophil granule proteases** generated fibrinogen-, fibrin- and CRP-degradation products with distinctly different apparent molecular mass values to

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* α_1-antitrypsin (α_1-antiproteinase) is a naturally occurring inhibitor of HNE.
** Referred to as lysosomal enzymes in previous studies.[1-3]

those generated by the neutrophil conditioned medium,[1-3] it appeared that this might have been due to discrepancies in protease concentrations. Granule enzyme preparations had been obtained by stimulating neutrophils with the chemotactic peptide FMLP in the presence of cytochalasin B, a process known to induce very high levels of degranulation by neutrophils.[8] In contrast, neutrophils stimulated with low dose PMA have been reported to release insignificant amounts of granule enzyme into the conditioned medium.[1,19,20] Thus, in order to compare the proteolytic activity of these two preparations, it is essential that serial dilutions of granule enzyme preparations are used. Indeed, the present study found that when sufficiently diluted, granule enzymes generated fibrinogen degradation products with apparent molecular mass values identical to those generated by the conditioned medium.

Investigation of the apparent molecular mass of the membrane associated proteolytic activity

Although it appeared that the proteolytic activity of the conditioned medium was due to proteases of the azurophil granule (M_r ±25-30 kDa), previous studies had identified this membrane associated proteolytic activity in regions of the gel corresponding to molecular mass values of 501, 396, 316 and 200 kDa.[1-3] In these studies the molecular size of this proteolytic activity was determined by eluting the enzyme activity from gel slices after separating concentrated enzyme preparations by SDS-PAGE.[1-3] In contrast, the present study employed zymography to determine the molecular weight of the membrane associated proteolytic activity. Due to the sensitivity of this technique, concentrating steps were not required prior to electrophoresis as in earlier studies.

Using casein as a substrate, zymography provided strong evidence that HNE was associated with the neutrophil cytoskeleton fraction. Not only did the proteolytic activity of the cytoskeleton preparation migrate to identical positions to that of purified HNE, but these bands were abolished following pre-incubation with MeO-Suc-$(Ala)_2$-Pro-Val-CH_2Cl. When samples were not boiled prior to electrophoresis, the proteolytic activities of both purified HNE and

cytoskeleton preparations were detected as a broad band in the 30 kDa area of the gel and a narrow band in the 69-97 kDa region. The latter probably represents a trimeric aggregate of HNE. It is interesting to note that AEBSF affinity chromatography (see chapter 4) isolated proteolytic activity from cytoskeleton preparations with electrophoretic mobility which appeared identical to that seen on zymograms of these unboiled preparations - compare figure 11 (page 145) to figure , lane 1, (chapter 4, page 119). It thus appears that AEBSF-affinity chromatography may well have been successful in isolating granule proteases from cytoskeleton preparations.

With respect to the proteolytic activity of the conditioned medium, fibrinogen zymography revealed proteolysis to be confined to the 30-60 kDa region of the gel. As the concentration of the sample was increased, activity extended higher up the gel, but never beyond 90 kDa. With the exception of one ultraconcentrated preparation (see later) there were no areas of activity in the regions of the gel previously reported to contain the subunits of the 600 kDa protease. In CRP zymograms, proteolytic activity was confined to the 30 kDa region of the gel. The zone of proteolysis between 30-60 kDa seen in fibrinogen zymograms is not incompatible with azurophil granule proteases. The reduced electrophoretic mobility of these proteases may have been attributed to two factors.

Firstly, in order to preserve proteolytic activity, samples are not boiled prior to zymography. Whilst enabling proteases to retain their catalytic activity, this does however, result in a tendency for proteins to aggregate.[11] Indeed, it has been noted that in most commercial enzyme preparations, proteolytic activity is associated with more than one band, and it has been suggested that this is most likely due to presence of catalytically active aggregates of enzymes, the catalytically active autodigestion products of these enzymes, or to the presence of other contaminating proteases.[21,22]

The tendency to form aggregates was, in fact, observed when the neutrophil cytoskeleton fraction, as well as commercially obtained HNE, were analysed in casein zymograms. In samples which had been boiled, the proteolytic activity was noted to be confined to the 25-30 kDa region of the gel. However, when the same samples did not undergo a boiling step prior to electrophoresis, a portion of the

proteolytic activity was present in the 69-97 kDa region, suggesting the formation of trimeric aggregates. This tendency to form aggregates, in the absence of boiling, may explain the extension of proteolytic activity observed beyond the 30 kDa area in fibrinogen containing zymograms. However, this would not explain the confinement of proteolytic activity to the 30 kDa area in CRP zymograms.

A second possible explanation is that the inclusion of protein substrates within gels affects the migration of proteases within the polyacrylamide gel. This would, however, be expected to affect the migration of protein standards as well.[11] It is possible, however, that affinity reactions between the protease and the co-polymerised substrate may decrease the electrophoretic mobility of certain proteases. Indeed it was noted that whilst the proteolytic activity of the cytoskeleton fraction migrated to the 25-30 kDa region of the gel in casein zymograms, the proteolytic activity of the same preparation migrated as a band corresponding to approximately 50 kDa in fibrinogen zymograms. Two factors suggest that the 50 kDa band in fibrinogen zymograms represented the same proteolytic activity as that associated with the 25-30 kDa band in casein zymograms.

Firstly, casein is widely acknowledged as a universal substrate,[12] and is readily degraded by almost all proteases. Therefore, if the band in the 50 kDa area of the fibrinogen zymogram had represented a distinct protease, it would have been observed in a similar region in casein zymograms. Secondly, there was strong evidence to suggest that the band in the 25-30 kDa region of casein zymograms of cytoskeleton preparations was HNE (see above). As HNE is known to degrade fibrinogen, it should be detected in fibrinogen zymograms as well. Therefore the absence of such a band in the 25-30 kDa area in fibrinogen zymograms, in the face of a solitary band in the 50 kDa region, suggested that the latter most likely represented HNE. Therefore, reduced migration of azurophil granule proteases in fibrinogen containing gels may offer the more likely explanation for the extension of the proteolytic activity beyond the 30 kDa area.

These findings confirm that the proteolytic activity of the conditioned medium and cytoskeleton preparations is associated with proteases of low molecular weight. The previously reported

association of proteolytic activity with high molecular weight regions of the gel,[1-3] is most likely attributable to aggregates of granule proteases, which have a tendency to form during preparative procedures, in particular following lyophilisation. It was indeed noted during the present study, that in an ultraconcentrated preparation containing a large amount of insoluble material, proteolytic activity extended into the high molecular weight regions of the gel, and a discrete area of proteolysis was noted at the very top of the gel. In samples that did not undergo these concentrating steps, however, this proteolytic zone was never observed.

In summary, on the basis of inhibitor profiles, substrate degradation patterns and molecular weight, this series of experiments provided strong evidence to suggest that the proteolytic activity previously ascribed to a novel 600 kDa protease was in fact due to proteases of the azurophil granule, predominantly HNE and cathepsin G.

The origin of granule proteases in the three neutrophil fractions

Recent studies suggest several mechanisms whereby neutrophil membrane, cytoskeleton and conditioned medium fractions may come to be inhabited by azurophil granule proteases. The origins of these proteases in each neutrophil fraction are considered in turn below.

1. Conditioned medium

As azurophil granules represent the least mobilisable subset of the neutrophil granules, their constituents have traditionally been considered to play less of a role extracellularly than those of other granule subsets.[23,24] More recently, however, neutrophils have been shown to express azurophil granule proteases on their external membranes in response to a variety of stimuli (including PMA used at a concentration as low as 0.4ng/ml[13]).[4-7,13] This implies the release of azurophil granules. It appears that neutrophils can release azurophil granule proteases even in the absence of exogenous stimuli. This is suggested by the recent observation that neutrophils, maintained at 37°C in fibronectin coated wells, periodically release

individual azurophil granules with accompanying bursts of proteolytic activity.[25] It is well known that neutrophil isolation procedures can cause a degree of neutrophil activation,[26,27] and this may contribute to azurophil granule release during subsequent incubation at 37°C. As the azurophil granule proteases are stored at high concentrations,[25,26] a relatively minor release of these granules can be associated with a significant increase in proteolytic activity.[25] Recent studies therefore suggest that the conditioned medium of PMA (5-10ng/ml) stimulated neutrophils would be expected to contain azurophil granule proteases.

2. Neutrophil membrane

As mentioned above, several studies have demonstrated the expression of both HNE and cathepsin G on the neutrophil membrane.

Whilst earlier studies appeared to exclude the presence of azurophil granule proteases in isolated neutrophil membrane fractions by testing for the presence of azurophil granule markers such as myeloperoxidase and β-glucuronidase,[19,20] recent evidence suggests that this may not be a reliable approach. Both HNE and cathepsin G are highly cationic proteins which bind readily to the neutrophil membrane by a charge related mechanism.[4,6,28] This propensity for charge related interactions is not necessarily shared by the commonly employed azurophil granule markers. For instance, it has been shown that the neutrophil plasma membrane has a very high capacity to bind HNE and cathepsin G in comparison to the azurophil granule marker myeloperoxidase.[4] It is possible that during membrane isolation procedures (which involve neutrophil lysis as an initial step), liberated HNE and cathepsin G may bind to solubilised neutrophil membranes, whilst the frequently used azurophil granule markers exhibit minimal binding. In this way neutrophil membrane preparations reported to be free of granule markers, may in fact contain azurophil granule proteases.

Recent reports indicate that following subcellular fractionation, neutrophil membranes contain a significant proportion of total cellular content of HNE.[13] Membranes isolated from unstimulated

neutrophils have been reported to contain 14% of the total neutrophil HNE activity, and 20% in neutrophils incubated for 5 minutes at 37°C prior to subcellular fractionation.[13]

3. Neutrophil cytoskeleton

As with neutrophil membranes, the cytoskeleton fraction is prepared from neutrophil lysates.[29] Following solubilisation of neutrophils with triton-X-100, it is possible that azurophil granule proteases may become associated with the triton-insoluble cytoskeleton via ionic interactions. It is worth noting that the solution which is subsequently used to elute proteins from the insoluble cytoskeleton is of the same ionic strength (1M KCl) as that which has previously been reported to elute HNE from neutrophil membranes.[6] This may explain the presence of azurophil granule proteases in preparations of solubilised cytoskeleton proteins.

Azurophil granule protease aggregation

The cationic serine proteases of the azurophil granule have a tendency to form heteroaggregates with anionic serine/glycine proteoglycans.[28] It has been suggested that such aggregation may play an important role in the packaging of neutrophil granules. Alterations in protein concentration, ionic strength and pH may all trigger aggregation of these proteases.[28] Whilst complexes of aggregated proteins usually dissociate following granule release,[25] serine proteases, still complexed to proteoglycans, have been reported to be exocytosed from rat mast cells.[30]

The tendency for azurophil granule proteases to form heteroaggregates with proteoglycans might explain their detection as high molecular weight proteolytic activity in earlier studies.[1-3] Finally, the formation of heteroaggregates with proteoglycans would explain the failure of this proteolytic activity to stain with coomassie blue, as proteoglycans are resistant to such staining.

Summary and conclusions

This study provides strong evidence to suggest that the proteolytic activity previously ascribed to a novel 600 kDa membrane associated protease is, in fact, due to the concerted effort azurophil granule proteases, predominantly HNE and cathepsin G. Thus the important observations in previous studies that the neutrophil membrane can degrade fibrinogen, fibrin and CRP with numerous consequences both for inflammation and coagulation can now be linked to these proteases. This is particularly interesting in view of the recent studies indicating that membrane bound azurophil granule proteases, through their abilility to evade naturally occurring protease inhibitors, may represent the biologically relevant forms of these proteases.[4-7]

References

1. Shephard EG, Kelly SL, Anderson R, Fridkin M. (1992) *Clin. Exp. Immunol.* **87**, 509.
2. Kelly SL, Adams SA, Robson SC, Kirsch RE, Shephard EG. (1994) *Biochem. J.* **298**, 689.
3. Adams SA, Kelly SL, Kirsch RE, Robson SC, Shephard EG. (1995) *Blood Coag. Fibrinol.* **6**, 693.
4. Bangalore N, Travis J. (1994) *Biol. Chem. Hoppe Seyler.* **375**, 659.
5. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. (1995) *J. Cell Biol.* **131**, 775.
6. Owen CA, Campbell MA, Boukedes SS, Campbell EJ. (1997) *Am. J. Physiol.* **272**, L385.
7. Salcedo R, Wasserman K, Patarroyo M. (1997) *Exp. Cell. Res.* **233**, 33.
8. Shephard EG, Anderson R, Beer SM, Jansen van Rensburg CE, De Beer FC. (1988) *Clin. Exp. Immunol.* **73**, 139.
9. Kelly SL. (1995) A neutral protease of the neutrophil surface: Role in the proteolyis of C-reactive protein and fibrinogen.(PhD Thesis, University of Cape Town)
10. Laemmli UK. (1970) *Nature.* **227**, 680.
11. Lantz MS, Ciborowski P. (1994) *Meth. Enzymol.* **235**, 563.
12. Reimerdes EH, Klostermeyer H. (1976) *Meth. Enzymol.* **45**:26.
13. Gaudin P, Berthier S, Barro C, Zaoui P, Morel F. (1997) *Eur. J. Cell. Biol.* **72**, 345.
14. Weitz JI, Huang AJ, Landman SL, Nicholson SC, Silverstein SC (1987) *J. Exp. Med.* **166**, 1836.
15. Powers JC, Odake S, Oleksyszyn J, Hori H, Ueda T, Boduszek B, Kam C. (1993). *Agents Actions Suppl.* **42**, 3.

16. Csernok E, Ernst M, Schmitt W, Bainton DF, Gross WL. (1994) *Clin. Exp. Immunol.* **95**, 244.
17. Weitz J, Landman SL, Crowley KA, Birken S, Morgan FJ. (1986) *J. Clin. Invest.* **78**, 155.
18. Weitz JI, Silverman EK, Thong B, Campbell EJ. (1992) *J. Clin. Invest.* **89**, 766.
19. Shephard EG, Beer SM, Anderson R, Strachan AF, Nel AE, De Beer FC. (1989) *J. Immunol.* **143**, 2974.
20. Pontremoli S, Melloni E, Michetti M, Sacco O, Sparatore B, Salamino F, Damiani G, Horecker BL. (1986) *Proc. Natl. Acad. Sci. USA.* **83**, 1685.
21. Lacks SA, Springhorn SS. (1980) *J. Biol. Chem.* **255**, 7467.
22. Keil-Dlouha V. (1976) *Biochim. Biophys. Acta.* **429**, 239.
23. Borregaard N, Lollike K, Kjeldsen L, Sengelov H, Bastholm L, Nielsen MH, Bainton DF. (1993) *Eur. J. Haematol.* **51**, 187.
24. Sengelov H, Follin P, Kjeldsen L, Lollike K, Dahlgren C, Borregaard N. (1995) *J. Immunol.* **154**, 4157.
25. Liou TG, Campbell EJ. (1996) *J. Immunol.* **157**, 2624.
26. Berger M, O' Shea J, Cross AS, Folks TM, Chused TM, Brown EJ, Frank MM. (1984) *J. Clin. Invest.* **74**, 1566.
27. Fearon DT, Collins LA. (1983) *J. Immunol.* **130**, 370.
28. Gullberg U, Andersson E, Garwicz D, Lindmark A, Ohlsson I. (1997) *Eur. J. Haematol.* **58**, 137.
29. Yassim R, Shefayk J, White JR, Tao W, Volpi M, Molsk TFP, Nacacche PH, Feinstein MB, Sharafi RI. (1985) *J. Cell Biol.* **101**, 182.
30. Serafin WE, Katz HR, Austin FK, Stevens RL. (1986) *J. Biol. Chem.* **261**, 15017.

Chapter Six

The localisation of neutrophil associated fibrinogen products

A reported consequence of neutrophil mediated fibrinogenolysis is the association of low molecular weight fibrinogen degradation products (FDP) with the neutrophil (figure 1).[2] These neutrophil associated products, which are all less than 30 kDa in size, have been shown to inhibit neutrophil adherence to immobilised fibrinogen.[2] The partial inhibition of [^{125}I]-labelled FDP binding to neutrophils by antibodies directed against various β_2-integrin receptors, led to the suggestion that these products, could inhibit β_2-integrin dependent functions, and may thus modulate neutrophil responses at sites of inflammation.[2] (For a review of β_2-integrin receptors see chapter 2, pages 38-41).

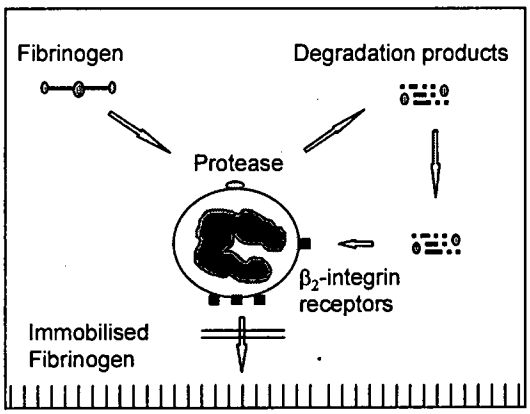

Figure 1. *Fibrinogen proteolysis by a neutrophil membrane protease was reported to generate low molecular weight FDP which associate specifically with neutrophil β_2-integrin receptors, thereby blocking neutrophil adhesion to immobilised fibrinogen. It was suggested that these products might modulate other β_2-integrin dependent functions as well.*[2]

The present study aimed to confirm the relationship between FDP and β_2-integrin receptors, using immunoelectron microscopy to co-localise these molecules on the neutrophil surface.

Methods

Chemicals and Reagents

The sources of chemicals and reagents used in this study are listed in *Appendix 1*, page 225-226.

Neutrophil isolation and preparation and iodination of fibrinogen.

These procedures were performed as described previously (see chapter 4, *Methods*, page 107-108). Purified neutrophils were resuspended in either phosphate buffered saline (PBS) or Hanks balanced salt solution (HBSS), and kept on ice until the beginning of experimental procedures.

Biotinylation of fibrinogen and BSA

Fibrinogen (6mg/ml) in 0.1M sodium bicarbonate buffer (0.1M $NaHCO_3$, 0.1M NaCl, pH=8.2) was added to biotinamidocaproic acid-3-sulpho-N-hydroxysuccinaminde (NHS) ester* (1.92mg) in a volume of 3ml. This represents a molar ratio of biotin to fibrinogen of approximately 65:1. The mixture was allowed to rotate end-on-end at room temperature for 60 minutes after which it was dialysed at 4°C against PBS (3 x 1 litre) over 16 hours. The biotinylated fibrinogen was divided into aliquots and stored at -70°C until use. Biotinylated BSA was prepared in a similar manner. Biotinylation of these proteins was confirmed by dot blotting using the biotin streptavadin detection system.

*Biotinamidocaproic acid-3-sulpho-N-hydroxysuccinaminde (NHS) ester is a water soluble NHS ester of biotin which has a 7 carbon atom spacer arm.

Neutrophil experiments for immunoelectron microscopy

Neutrophil β_2-integrin expression

Neutrophils (2 x 10^6/ml) in PBS were warmed to 37°C for 10 minutes, after which they were stimulated with fMLP (1μM) for a further 15 minutes. Neutrophils were then centrifuged (2000rpm, 5 minutes, 4°C) and following a washing step at 4°C in PBS containing 0.5% BSA (PBS/A), cells were immunostained with monoclonal antibodies directed against CD11b, diluted 1:5 in PBS/A for 1 hour on ice. Control samples were stained with non-immune mouse IgG used at the same concentration. Neutrophils were then washed 3 times with PBS/A and fixed with a combination of 3.5% paraformaldehyde and 0.2% glutaraldehyde in 0.1M phosphate buffer (pH=7.4) for 1 hour at 4°C in preparation for ultracryotomy (see below).

Neutrophil association with FDP

Neutrophils (2 x 10^6/ml) in PBS (or HBSS) were warmed to 37°C for 10 minutes, and then stimulated with PMA (5ng/ml). Immediately following the addition of PMA, biotinylated or unlabelled fibrinogen (1-2.5 mg/ml) was added to the neutrophils and the incubation was continued for various time intervals (5-60 minutes, 37°C). (During this incubation fibrinogen is degraded by the neutrophil generating a variety of high and low molecular weight FDP).[2] At selected time points neutrophils were centrifuged (2000rpm, 5 minutes, 4°C), washed 3 times with ice cold PBS/A, and fixed (as above) in preparation for ultracryotomy.

In some experiments neutrophils were immunostained prior to fixing: Following incubation with fibrinogen neutrophils were washed (as above) and then incubated with rabbit anti-human fibrinogen antibody (0.5 mg/ml) in PBS/A for 1 hour on ice. Cells were then washed 3 times and fixed as above. Controls were included which had been exposed to the same concentration of non-immune rabbit IgG. Staining with the secondary antibody was performed following ultracryotomy.

Neutrophil association with FDP and biotinylated BSA

Neutrophils (2 x 10^6/ml) were stimulated with PMA as described above prior to the addition of biotinylated BSA (1mg/ml) and fibrinogen (1mg/ml). Neutrophils were then incubated at 37°C for various time intervals (5-60 minutes), after which they were centrifuged, washed and fixed as above in preparation for ultracryotomy.

Ultracryotomy and immunostaining

Following fixation, cells were washed once with PBS, embedded in 2% low melting point agarose, infiltrated with 2.3 M sucrose and cryosectioned at -100 to -110°C.[3] Ultrathin sections were retrieved with a droplet of 2.3 M sucrose suspended on a wire loop, and transferred to formvar coated nickel grids. Each grid was floated on PBS for at least ten minutes to allow for the diffusion of sucrose. Grids were then transferred to a droplet of 2% (w/v) gelatin for 10 minutes, after which they were placed on droplets of 0.02M glycine (3 x 1 minute incubations). Each grid was transferred to a droplet of 1% BSA in PBS for 1 minute, prior to immunostaining at room temperature.*

Detection of β_2-integrins

As neutrophils had been immunostained with primary antibody (against CD11b and CD18) prior to sectioning, post sectioning staining involved only the use of a secondary antibody. Sections were stained with 30nm-gold conjugated goat-antimouse IgG, diluted 1:20 in PBS containing 1% BSA (1% PBS/A), overnight in a humidified chamber.†

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* All procedures described below represent those which provided the optimal results for each particular approach. A number of conditions were varied during optimisation procedures, including fixation, antibody concentrations, duration of immunostaining and the composition of the incubation buffer.

† Whilst this procedure was established for the detection of CD11b/CD18, the intention was to apply this procedure to the detection of CD11a/CD18 and CD11c/CD18 as well. However, for reasons discussed later (see results), this was not ultimately required.

Detection of biotinylated FDP

For the detection of biotinylated FDP, three detection systems were tried. These are listed below: (All incubations were performed at room temperature)

- **Streptavadin gold.** Sections were stained with 10nm-gold conjugated streptavadin (diluted 1:20 in PBS/A) overnight in a humidified chamber.
- **Streptavadin biotin bridge gold technique.** Grids were floated on blocking buffer (1% BSA, 0.01% Tween 20, 0.5M NaCl in PBS) for 30 minutes prior to staining with streptavadin for 30 minutes. Following washing (10 x 2 minute washes on blocking buffer), sections were incubated with biotinylated BSA conjugated to 10nm colloidal gold (bBSA-gold) diluted 1:50 for 30 minutes.
- **Antibiotin antibody.** Sections were stained with 15nm-gold conjugated goat antibiotin antibody (diluted 1:20 in 1% PBS/A) overnight in a humidified chamber.

Detection of unlabelled FDP

Sections were stained with rabbit anti-human fibrinogen antibody or non-immune rabbit IgG (2μg/ml) in 1% PBS/A overnight and then washed (10 x 2 minute washes on 1% PBS/A), before staining with 5nm-gold conjugated goat antirabbit IgG for 1 hour in the same buffer.

Detection of biotinylated BSA

Sections were stained with goat antibiotin antibody conjugated to 15nm gold diluted 1:20 in 1% PBS/A overnight in a humidified chamber.

Following immunostaining, sections were washed with 5 drops 1% PBS/BSA over a period of 10 minutes, after which they were washed with 5 drops of PBS over the same period. Sections were then post-

fixed on a drop of 1% glutaraldehyde in PBS for 5 minutes, after which they were washed with PBS (2 drops, 5 minutes each), followed by distilled water (5 drops, 2 minutes each). Sections were then stained for 10 minutes with neutral uranyl acetate (2%) and washed briefly with distilled water (2 drops, 20 seconds each), prior to staining with acidic uranyl acetate (2%) in methylcellulose (1.8%) (2 drops, 5 minutes each). Grids were retrieved with a wire loop and air dried prior to viewing under the electron microscope.

Association of [^{125}I]-labelled fibrinogen and BSA with neutrophils

Neutrophils (5 x 10^6/ml) were warmed to 37°C for 10 minutes prior to stimulation with PMA (5ng/ml). Immediately following stimulation, [^{125}I]-labelled fibrinogen or [^{125}I]-labelled BSA were added at various concentrations and for various durations of time as indicated in the results section. At selected time points, cells were placed on ice and washed three times with 0.5% PBS/A at 4°C. Neutrophils were then transferred to clean tubes and the associated radioactivity was measured in a gamma counter. These experiments were performed using PBS, HBSS and HEPES buffered HBSS as incubation buffers. When necessary, adaptations were made to the basic experimental procedure and these are listed below.

Release of internalised [^{125}I]-labelled fibrinogen

To determine whether [^{125}I]-labelled fibrinogen internalised by the neutrophil was subsequently released, PMA (5ng/ml) stimulated neutrophils were incubated with [^{125}I]-labelled fibrinogen for 10 minutes at 37°C, and following washing steps (as above), cells were resuspended in HBSS containing PMA (5ng/ml), and incubated at 37°C for various periods of time. The radioactivity released into the supernatant was measured and expressed as a percentage of total neutrophil associated fibrinogen (measured following washing steps). Viability, as determined by trypan blue exclusion, was greater than 90% at the end of the experiment. The supernatants were lyophilised, reconstituted in SDS sample buffer, and subjected to SDS-polyacrylamide gel electrophoresis and autoradiography.

Association of [^{125}I]-labelled ligand at 4°C.

Neutrophils were stimulated with PMA at 37°C for 30 minutes* (as above), placed on ice for a further 30 minutes, after which they were incubated in the presence of [^{125}I]-labelled fibrinogen or BSA, on ice, for various periods of time (see results). Identical experiments were also performed using [^{125}I]-labelled fibrinogen degradation products (see below). For these experiments, BSA in the washing buffer was replaced with an equal concentration of casein.**

Effect of BSA on [^{125}I]-labelled fibrinogen association

In experiments where the effect of BSA on [^{125}I]-labelled fibrinogen association with the neutrophil was investigated, neutrophils were pre-incubated in the presence of BSA (2mg/ml) for 10 minutes prior to the addition of [^{125}I]-labelled fibrinogen. Control samples received an equal volume of assay buffer. Experiments were allowed to proceed as described above.

Generation of [^{125}I]-labelled fibrinogen degradation products

PMA stimulated neutrophil conditioned medium was prepared as described earlier (Chapter 3, *Methods*, page 108). [^{125}I]-labelled fibrinogen (2mg) was incubated with conditioned medium (from 15 x 10^6 neutrophils) in a final volume of 2ml for 24 hours at 37°C. The percentage TCA solubility of [^{125}I]-labelled fibrinogen degradation products prepared in this way exceeded 50%.

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*Neutrophils were stimulated at 37°C to allow the membrane phenotype of the neutrophil to change in response to PMA. Neutrophils were subsequently cooled in order to inhibit the endocytic apparatus of the neutrophil prior to exposure to radiolabelled ligand.

**As these studies were testing for *surface* binding of fibrinogen and BSA, the washing buffer could not contain BSA as it would compete with [^{125}I]-labelled BSA during washing steps, potentially removing surface associated [^{125}I]-labelled BSA. As a result BSA in the washing buffer was replaced with casein used at the same concentration.

Results

Immunoelectron microscopy

Expression of β_2-integrins

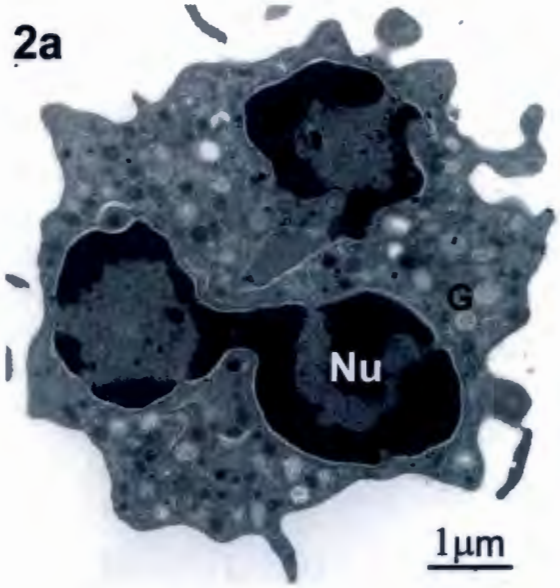
In order to detect β_2-integrin receptors, two adaptations to standard resin embedding procedures were made. It is well known that certain antigens, particularly those associated with the cell membrane, are inaccessible to antibodies when embedded in resin,[4] and this proved to be the case for β_2-integrins (Figure 2a). Thus, the ultracryotomy approach of Tokayasu, which involves the cutting of thin sections from samples hardened by freezing rather than resin embedding, was implemented.[3] Despite the use of this technique, β_2-integrin receptors were still not detectable on the neutrophil surface. As the sensitivity of certain antigens to aldehyde fixation is well documented,* immunostaining with the primary antibody prior to aldehyde fixation and cryosectioning was attempted (see *Methods*).[5]

Using this procedure, membranes of activated neutrophils stained positively for the CD11b component of the β_2-integrin receptor, CD11b/CD18 (figure 2b). The specificity of this procedure was confirmed by the absence of labelling when primary antibodies were substituted with non-immune control antibodies (figure 2c). In contrast to stimulated cells, unstimulated cells showed sparse labelling of their plasma membranes. A similar pattern of staining was obtained using antibodies directed against the CD18 subunit of this receptor (not shown).

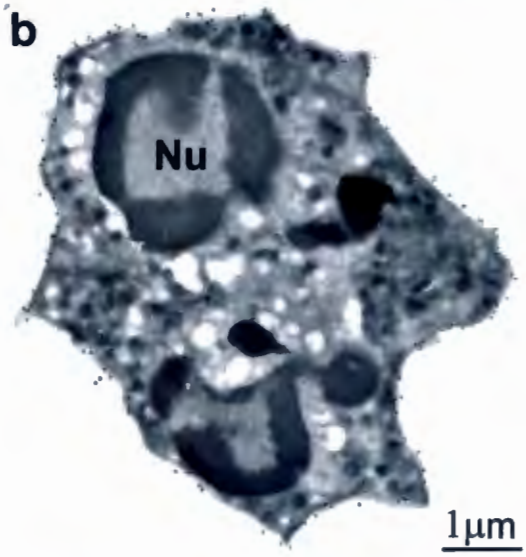
Cell associated fibrinogen degradation product (FDP)

Irrespective of the detection system used, FDP were not detected on the surface of either resting or PMA stimulated neutrophils incubated

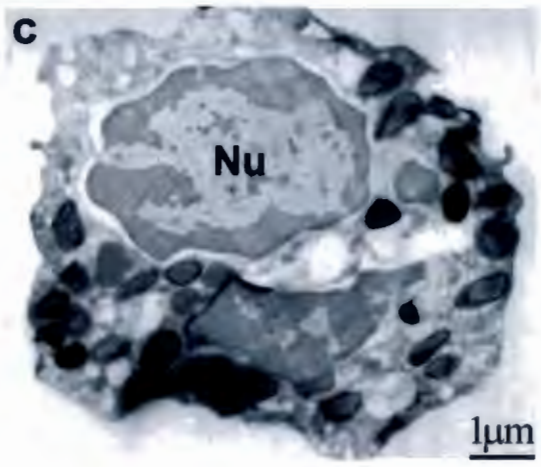
* This is particularly apparent when monoclonal antibodies are used.



(a) A resin section of an FMLP stimulated neutrophil. Resin embedding is generally associated with superior ultrastructural preservation of cells compared to cryo-sectioning. This micrograph shows the basic structure of the neutrophil with its multilobed nucleus (Nu) and its cytoplasm densely packed with granules (G). Immuno-staining for CD11b/CD18 was unsucessful in resin embedded neutrophils.*



(b) A cryosection of an FMLP stimulated neutrophil immunostained for CD11b/CD18. Neutrophils underwent pre-fixation staining with anti-CD11b monoclonal antibody. This antibody was detected on cryosections using 30nm-gold conjugated goat anti-mouse IgG (see Methods). Numerous gold particles can be seen lining the surface of this neutrophil indicating the presence of CD11b/CD18 receptors.*



(c) To confirm the specificity of the immuno-labelling procedure (above) for CD11b, a control was included in which anti-CD11b antibody was substituted with non-immune mouse IgG (see Methods). No gold particles were detected on the neutrophil surface in these samples.

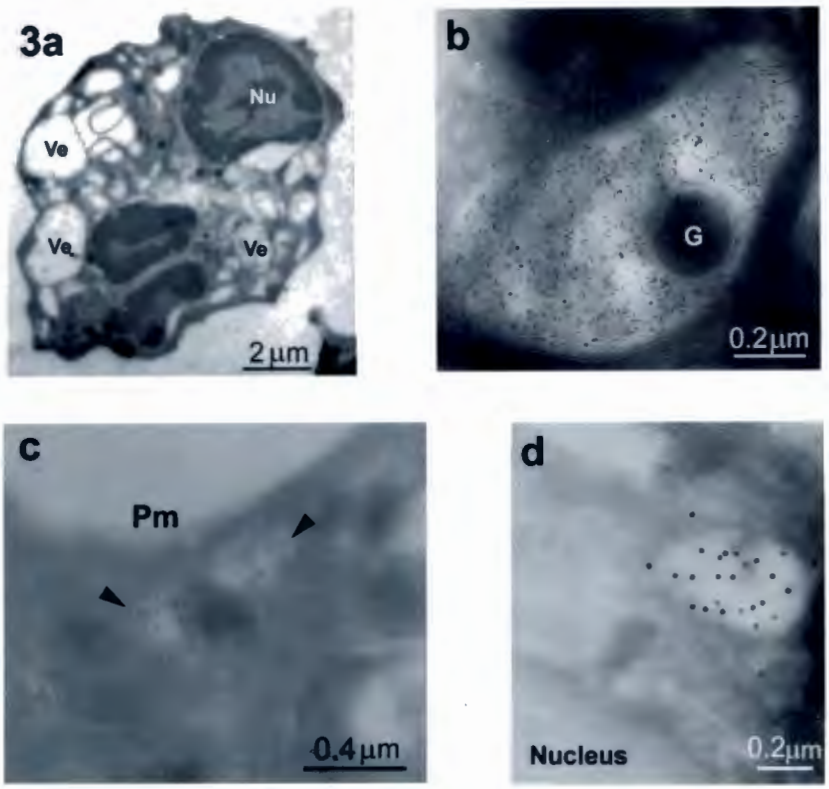


Figure 3. The immunolocalisation of fibrinogen products within intracellular vesicles of PMA stimulated neutrophils. *(a) A cryosection of a PMA (5ng/ml) stimulated neutrophil (30 minutes, 37 degrees C) showing multiple vesicles (Ve) in the cytoplasm. (b) An intracellular vesicle of a PMA stimulated neutrophil incubated with fibrinogen (2.5 mg/ml, 30 minutes, 37 degrees C). Fibrinogen was detected using rabbit antihuman fibrinogen antibody followed by 5nm-gold conjugated goat antirabbit IgG (see Methods). (c) Intracellular vesicles of a PMA stimulated neutrophil incubated with biotinylated fibrinogen (1.5 mg/ml, 30 minutes, 37 degrees C). Biotinylated fibrinogen was detected using the streptavadin-biotin bridge-gold (SBBG) technique (see Methods), (Pm=plasma membrane). (d) An intracellular vesicle of a PMA stimulated neutrophil incubated with biotinylated fibrinogen (1.75 mg/ml, 30 minutes, 37 degrees C). Biotinylated fibrinogen was detected using 15nm-gold conjugated goat antibiotin antibody (see Methods).*

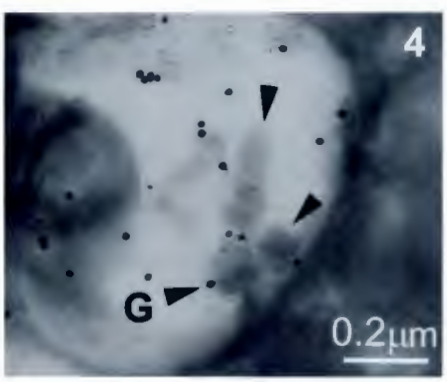


Figure 4: The co-localisation of fibrinogen and biotinylated BSA within an intracellular vesicle. *PMA (5 ng/ml) stimulated neutrophils were incubated with fibrinogen (1mg/ml) and biotinylated BSA (1mg/ml) for 30 minutes at 37 degrees C. Cryosections were immunostained for fibrinogen using rabbit antihuman fibrinogen antibody followed by 5nm-gold conjugated goat antirabbit IgG. Biotinylated BSA was detected using 15nm-gold conjugated antibiotin antibody (see Methods). Cytoplasmic granules can be seen within this vesicle (G, arrows).*

with fibrinogen (1-2.5 mg/ml) at 37°C over a time course of 5-60 minutes. FDP, were however, detected intracellularly within electron lucent vesicles (Ve) which were abundant in PMA stimulated neutrophils (Figures 3a-d). Indirect immunogold labelling (figure 3b) consistently provided the highest levels of intracellular labelling. Labelling was specific for fibrinogen as no labelling was observed when rabbit anti-human fibrinogen antibody was substituted with non-immune rabbit IgG. When ultrathin sections of neutrophils (which had been incubated with biotinylated fibrinogen) were stained with streptavadin conjugated to 10nm gold, no specific labelling was observed, despite a variety of adjustments to conditions of fixation as well as immunostaining (not shown). Two alternative approaches for the detection of biotinylated FDP were also pursued, namely the streptavadin-biotin bridge-gold (SBBG) technique, as well as the use of gold conjugated antibiotin antibody.* Whilst the SBBG technique was unable to detect biotinylated FDP at the neutrophil surface, a low level of labelling was noted intracellularly within electron lucent vesicles (figure 3c), but this was relatively weak and poorly reproducible. However, when (15nm) gold conjugated antibiotin antibody was used (figure 3d), labelling within electron lucent vesicles was consistent and reproducible. No surface labelling was detected using either of these techniques, despite various adjustments to the conditions of fixation and labelling (including pre and post-fixation labelling as well as adjustments to concentration and duration of exposure to gold conjugates). Increasing the concentration of fibrinogen as high as 2.5 mg/ml did not change the level of surface labelling.

The indirect immunogold labelling approach was used to investigate the uptake of FDP over a time course of 5-60 minutes. The intracellular accumulation of fibrinogen was time dependent and

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* A review of related studies revealed that the use of gold conjugated streptavadin is often associated with poor labelling due to steric hinderence.[6-9] Two alternative approaches were suggested to be more sensitive and specific for the detection of biotinylated ligands. The first, known as the streptavadin-biotin-bridge-gold (SBBG) technique,[6] is a two step staining procedure in which sections are first stained with streptavadin and thereafter with biotinylated BSA conjugated to colloidal gold. The second approach involves the use of a gold conjugated antibody directed against the hapten form of biotin.[7]

appeared to peak at about 30 minutes. In PMA stimulated neutrophils, gold labelled vesicles were plentiful and relatively densely labelled, whilst in unstimulated neutrophils these vesicles were much less frequently observed and were sparsely labelled. These findings were the same in neutrophils incubated in PBS and HBSS. These observations were consistent over a large number of cells in a particular section, over many sections and over many experimental samples. Thus the FDP that are associated with neutrophils[2] appear from these results to be intracellular rather than surface bound.

The next stage of this study aimed to determine the process by which FDP accumulated intracellularly. Since it may be possible that the previously documented neutrophil associated FDP[2] represent, at least in part, the intracellular degradation products of fibrinogen taken up in *intact* form, the text that follows will refer to uptake of *fibrinogen*, used as a general term to describe both intact and degraded forms of fibrinogen.*

Colocalisation of fibrinogen and biotinylated BSA

Because fibrinogen products appeared to accumulate intracellularly with no evidence of accumulation on the neutrophil surface, it was thought, initially, that fibrinogen uptake might occur via fluid phase pinocytosis.** To investigate this possibility, the pattern of fibrinogen uptake was compared to that of a frequently used marker of fluid phase pinocytosis, BSA.[10-16] It was reasoned that if fibrinogen uptake resembled that of BSA, then fibrinogen was most likely being internalised via a non-specific pinocytic pathway.

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* Under the experimental conditions employed, neutrophils degrade fibrinogen to yield both high and low molecular weight products.[1] In addition, intact fibrinogen, (not yet been degraded), is also present in the surrounding medium. Therefore under the experimental conditions employed, neutrophils will encounter intact fibrinogen as well as high and low molecular weight degradation products.

** Pinocytosis is the internalisation of liquid droplets by a cell through invaginations in the plasma membrane which close to form fluid filled vesicles. During this process molecules in the surrounding medium may be internalised without any significant adsorption to the plasma membrane.

Initial experiments investigated, using immunoelectron microscopy, the intracellular localisation of fibrinogen and biotinylated BSA (both at 1mg/ml) following incubation with PMA stimulated neutrophils. Neither fibrinogen nor bBSA accumulated on the neutrophil plasma membrane over a time course of 5-60 minutes. Both proteins consistently gained access to the same intracellular compartments as illustrated in figure 4.

Uptake of [^{125}I]-labelled fibrinogen and BSA

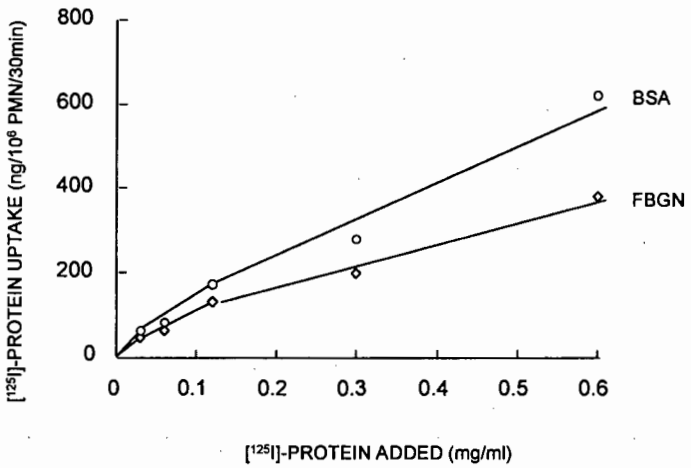
The co-localisation of fibrinogen and BSA within intracellular vesicles initially suggested that these proteins may be taken up via a similar pathway. However, a *quantitative* comparison of total associated protein (fibrinogen versus BSA) was required before this possibility could be entertained. Quantification was important in order to exclude the possibility that BSA was being taken up as an "innocent bystander" in the fluid at sites of specific receptor mediated uptake of fibrinogen. If this were the case, one would expect that the quantity of fibrinogen internalised by the neutrophil would greatly exceed that of BSA, as receptor mediated endocytosis involves selective concentration of the ligand on the neutrophil surface prior to internalisation.[17-19]

In order to compare the process of fibrinogen and BSA uptake, the [^{125}I]-labelled forms of these ligands were incubated with PMA stimulated neutrophils at 37°C.

The dose response curve for the uptake of [^{125}I]-labelled fibrinogen was found to be similar to that for [^{125}I]-labelled BSA (figure 5) using both PBS and HBSS as assay buffers. In view of the similar patterns of uptake displayed by fibrinogen and BSA, it was thought initially that the uptake of fibrinogen was occurring entirely via fluid phase pinocytosis. However, a prerequisite for fluid phase pinocytosis is that uptake varies linearly with the concentration of solute in the medium showing no evidence of saturation over a large (mg/ml) range.[17] The non-linearity of the curves below a concentration of 0.12 mg/ml suggested that the uptake of both fibrinogen and BSA might also involve an element of specific

binding to the neutrophil surface. This was unexpected in view of the frequent use of BSA as a marker of fluid phase pinocytosis.

5(a)



(b)

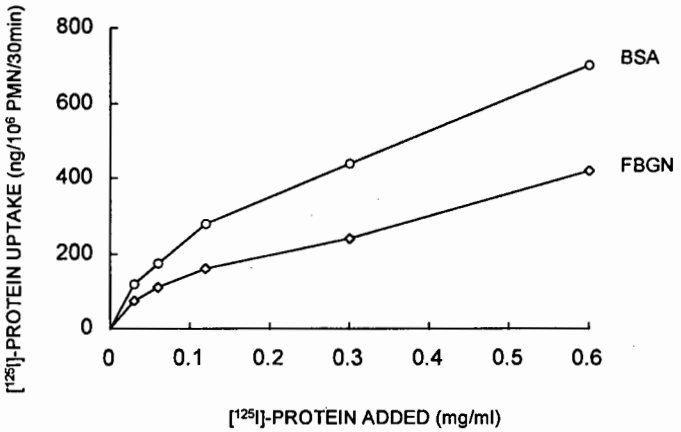


Figure 5: Association of [^{125}I]-labelled fibrinogen and BSA with PMA stimulated neutrophils (PMN), as a function of concentration. *(a) [^{125}I]-labelled fibrinogen (FBGN) or [^{125}I]-labelled BSA (0-600μg/ml) were incubated (37°C, 30 minutes) with PMA stimulated PMN (5 x 10^6/ml) in PBS. Association of [^{125}I]-labelled protein was measured and expressed as ng [^{125}I]-labelled protein per 10^6 PMN. Each data point represents a mean of duplicates. An additional experiment gave a similar result (appendix 3, page 232). (b) The same experiment was performed using HBSS as the assay buffer.*

[^{125}I]-labelled fibrinogen and BSA also displayed similar patterns of uptake with respect to time (performed in PBS) as illustrated in Figure 6.

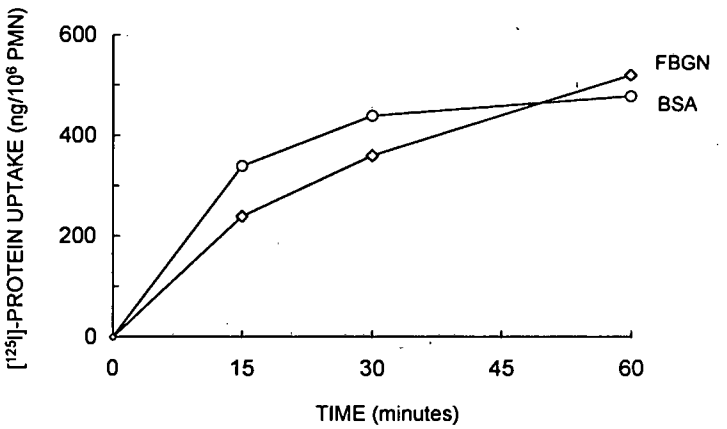


Figure 6: Association of [^{125}I]-labelled fibrinogen and BSA with PMA stimulated PMN as a function of time. *[^{125}I]-labelled fibrinogen or BSA (500μg/ml) were incubated with PMA stimulated PMN (5 x 10^6/ml) in PBS for varying durations of time (37°C, 15-60 minutes). Association of [^{125}I]-labelled protein was measured and expressed as ng [^{125}I]-labelled protein per 10^6 PMN. Each data point represents a mean of duplicates.*

Release of internalised fibrinogen

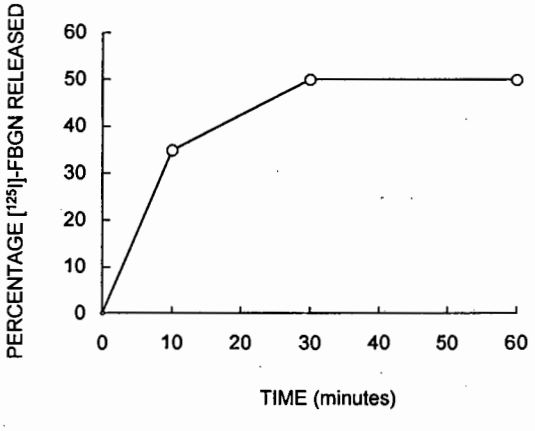
The observed similarities (both qualitative and quantitative) in the pattern of uptake of fibrinogen and BSA suggested that fibrinogen was being internalised predominantly by non-specific pinocytosis. As pinocytosis involves the bi-directional cycling of the plasma membrane,[17,20] the early release of internalised proteins back into the extracellular space would be expected. This has been well documented in neutrophils,[14] and various other cell types,[21-23] where a significant proportion of pinocytosed protein is rapidly released back into the surrounding medium, often in the form of catabolic products.[17]

To determine whether fibrinogen internalised by the neutrophil was subsequently released, PMA stimulated neutrophils were incubated

with [^{125}I]-labelled fibrinogen for 10 minutes and, following washing steps, were re-incubated at 37°C in the presence of PMA for various durations of time. The radioactivity released into the supernatant was measured and expressed as a percentage of the total radioactivity associated with the neutrophil at 10 minutes.

As illustrated in figure 7, neutrophils released a substantial proportion of internalised fibrinogen. This occurred relatively early on, with 35% of internalised fibrinogen being released within the first 10 minutes, 50% after 30 minutes, and thereafter minimal further release. A second experiment investigating release at earlier time points revealed that 30% and 31% of internalised fibrinogen were released at 5 and 10 minutes respectively. Viability at each time point was greater than 90% indicating that release of fibrinogen was not the result of cell death.

(7)



(8)

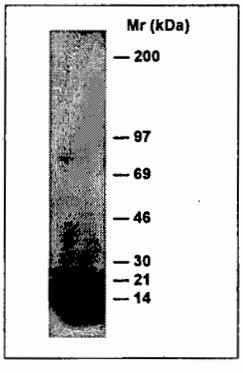


Figure 7: Release of neutrophil associated [^{125}I]-labelled fibrinogen. *PMA stimulated PMN were incubated with [^{125}I]-labelled fibrinogen (37°C, 10 minutes) as described. PMN were then washed three times at 4°C and re-incubated at 37°C in the presence of PMA for 10 to 60 minutes (see methods). The radioactivity released into the supernatant was counted and expressed as a percentage of total radioactivity associated with PMN at 10 minutes. Each data point represents a single measurement.*

Figure 8: SDS-PAGE analysis of the [^{125}I]-labelled fibrinogen products. *The [^{125}I]-labelled fibrinogen products released by the neutrophil after 15 minutes were subjected to SDS-PAGE and autoradiography.*

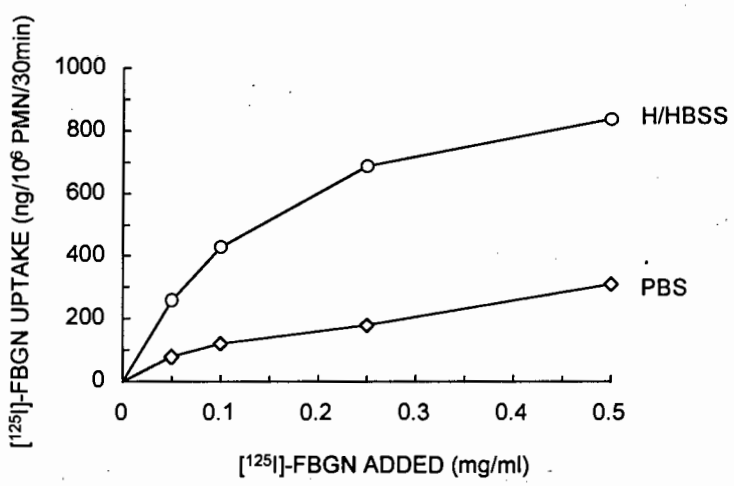
The [^{125}I]-labelled fibrinogen products released by the neutrophil at 15 minutes were analysed by SDS-PAGE and autoradiography (figure 8). These products were found to be of low molecular weight (mostly < 30kDa), resembling the [^{125}I]-labelled FDP previously reported to associate with PMA stimulated neutrophils.[2] No intact fibrinogen was present.

Divalent cations and uptake of [^{125}I]-labelled fibrinogen and BSA

As previous studies had investigated the association of [^{125}I]-labelled FDP with the neutrophil in both HBSS and PBS,[2] these buffers were used in the present study. Consistent with earlier reports,[2] uptake of [^{125}I]-labelled fibrinogen appeared similar in experiments performed in PBS (calcium free) and HBSS (which contains 1mM $CaCl_2$ and $MgCl_2$). During the present study, however, it was noted that HBSS was not able to maintain its pH at 7.4 for the duration of the assay, with the pH often rising to 7.8. Experiments were therefore repeated using (1g/l) HEPES (N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid) buffered HBSS. A marked increase in neutrophil association with both fibrinogen and BSA was noted when this buffer was used as illustrated in figure 9. This suggested that divalent cations might facilitate the association of fibrinogen and BSA with saturable sites on the neutrophil surface.

The association of fibrinogen (1mg/ml) with PMA stimulated neutrophils (37°C, 30 minutes) in HBSS-HEPES was analysed by immunoelectron microscopy. As in earlier experiments using PBS and HBSS, fibrinogen was detected intracellularly but not on the cell surface. All further experiments were performed using HEPES buffered HBSS as the assay buffer.

9(a)



(b)

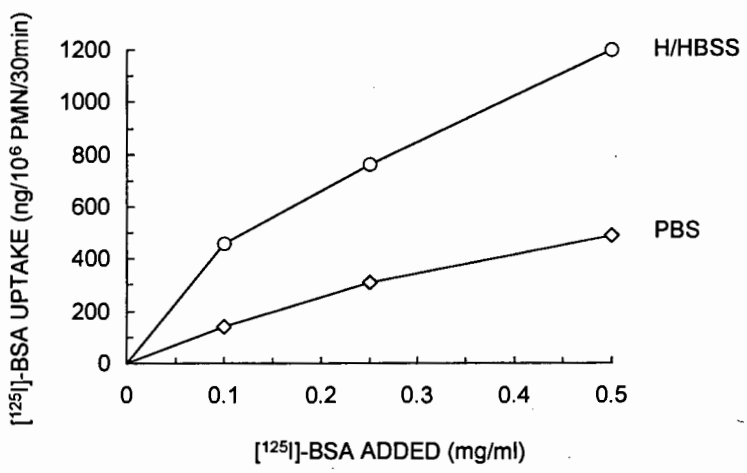


Figure 9: The effect of divalent cations on [^{125}I]-labelled fibrinogen and BSA association with PMN. *(a) [^{125}I]-labelled fibrinogen (0-500μg/ml) was incubated (37°C, 30 minutes) with PMA stimulated PMN (5 x 10^6/ml) in either PBS or HEPES buffered HBSS (H/HBSS). Association of [^{125}I]-labelled fibrinogen was measured and expressed as ng per 10^6 PMN. Each data point represents a mean of duplicates. An additional experiment gave similar results (appendix 3, page 232) (b) The association of [^{125}I]-labelled BSA with PMA stimulated neutrophils was tested under the same conditions.*

Temperature dependence of fibrinogen uptake

To investigate whether [^{125}I]-labelled fibrinogen and BSA could associate with the neutrophil surface, binding experiments were performed using cells chilled on ice (0°C). As endocytosis is completely inhibited at 0°C,[17] any association of [^{125}I]-labelled protein detected under these conditions, would suggest surface binding.

Neutrophil association with both [^{125}I]-labelled proteins was found to be markedly diminished in chilled cells but not abolished, suggesting that these proteins can bind to the neutrophil surface (Figures 10a and b). The association of [^{125}I]-labelled fibrinogen and [^{125}I]-labelled BSA with chilled neutrophils was also measured over a time course of 0-60 minutes. As indicated in figure 11, both proteins continued to associate with the neutrophil over the entire time course. An additional experiment investigating the association of [^{125}I]-labelled fibrinogen with the neutrophil at 0°C showed continued association over a time course of 0-90 minutes (appendix 3, page 233). These results suggest that this binding has a non-saturable component.

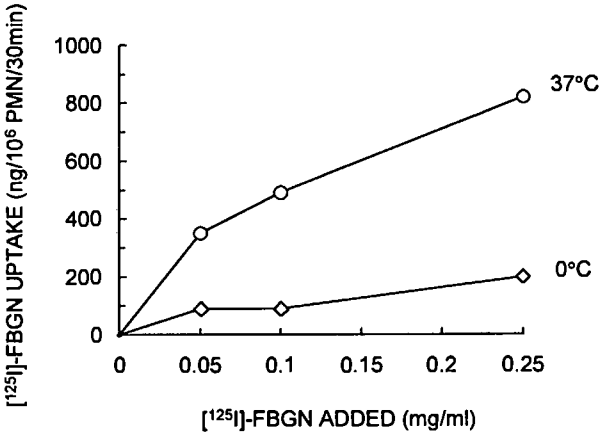


Figure 10a: Association of [^{125}I]-labelled fibrinogen with PMN at 0°C and 37°C. *PMA stimulated PMN (5 x 10^6/ml) in HEPES buffered HBSS were incubated (37°C, 30 minutes) with [^{125}I]-labelled fibrinogen (0-250μg/ml). In parallel experiments, PMA stimulated PMN (37°C, 30 minutes) were chilled on ice for 30 minutes, after which they were incubated with [^{125}I]-labelled fibrinogen for a further 30 minutes on ice. Association of [^{125}I]-labelled fibrinogen was measured and expressed as ng per 10^6 PMN. Each data point represents the mean of duplicates.*

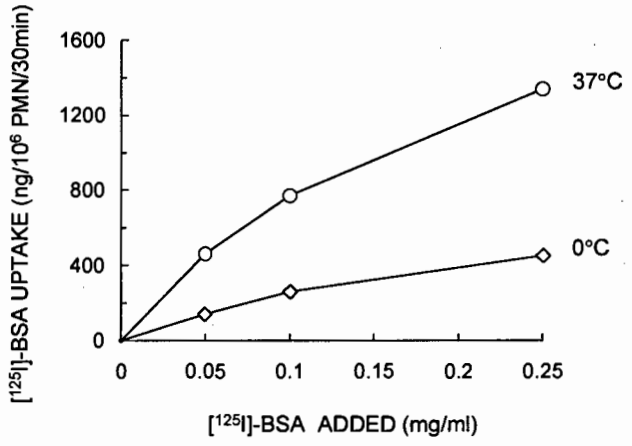


Figure 10b: Association of [^{125}I]-labelled BSA with PMN at 0°C and 37°C. *PMA stimulated PMN were incubated with [^{125}I]-labelled BSA (0-250μg/ml) at 0°C or 37°C as described for fibrinogen (figure 10a). Association of [^{125}I]-labelled BSA with PMN at 30 minutes was measured and expressed as ng per 10^6 PMN. Each data point represents the mean of duplicates.*

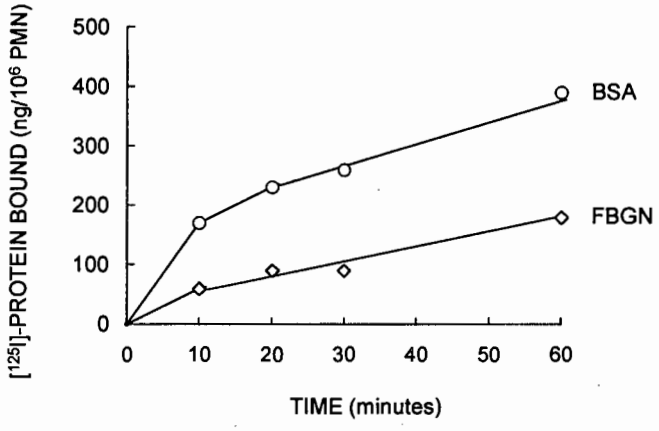


Figure 11: Association of [^{125}I]-labelled fibrinogen and BSA with chilled PMN as a function of time. *Chilled PMA stimulated PMN (prepared as described in figure 10) were incubated with [^{125}I]-labelled fibrinogen or BSA (100μg/ml) on ice over a time course of 0-60 minutes. Association of [^{125}I]-labelled protein was measured and expressed as ng per 10^6 PMN. Each data point represents the mean of duplicates.*

Neutrophil association with extensively degraded fibrinogen

Data obtained thus far suggested that the previously reported neutrophil associated FDP might not necessarily reflect the selective binding of low molecular weight FDP to the neutrophil as was previously thought.[2] It was equally likely that neutrophil associated FDP could occur through the *intracellular* degradation of internalised fibrinogen, taken up in intact and/or partially degraded forms.

To determine whether neutrophils showed a preference for binding degraded forms of fibrinogen, neutrophil association with extensively degraded fibrinogen was compared to association with intact fibrinogen at 0°C. Fibrinogen digests were generated by incubating [^{125}I]-labelled fibrinogen with the conditioned medium of PMA stimulated neutrophils as described in *Methods*. This generated a preparation in which over 50% of fibrinogen products had a molecular weight of ≤ 14 kDa (i.e. were soluble in 10% TCA).

PMA stimulated neutrophils were chilled as described earlier, after which they were incubated with either intact fibrinogen or fibrinogen digests (100μg/ml) for a period of 0-30 minutes. As illustrated in figure 12, the quantity of [^{125}I]-labelled product that became associated with the neutrophil appeared similar whether intact or degraded forms of fibrinogen were added to chilled neutrophils.

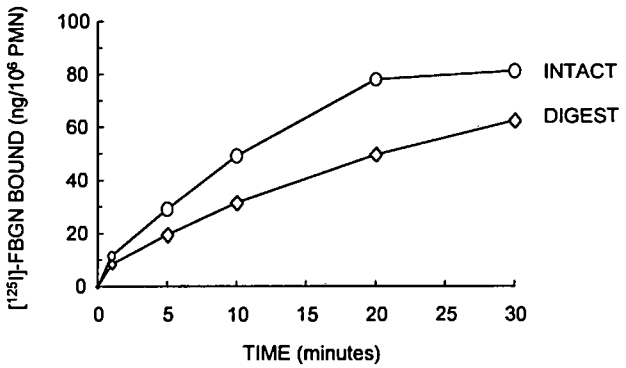


Figure 12: Association of [^{125}I]-labelled fibrinogen versus an [^{125}I]-labelled fibrinogen digest with chilled PMN. *Chilled PMA stimulated PMN were incubated with either [^{125}I]-FBGN (100μg/ml) or degraded [^{125}I]-FDP respectively (100μg/ml) over a time course of 0-30 minutes. Association of [^{125}I]-labelled product was measured and expressed as ng per 10^6 PMN. Each data point represents the mean of duplicates.*

The effect of BSA on fibrinogen uptake

Under all experimental conditions employed thus far, the pattern of fibrinogen uptake was consistently paralleled by BSA. This suggested that the mode of uptake of these proteins might be similar. To determine whether fibrinogen and BSA might compete for binding sites on the neutrophil surface, the ability of BSA to inhibit the uptake of [^{125}I]-labelled fibrinogen was investigated.

PMA stimulated neutrophils were incubated with [^{125}I]-labelled fibrinogen (0-500μg/ml) for 30 minutes at 37°C. Parallel samples were pre-incubated with BSA (2mg/ml) for 10 minutes prior to the addition of [^{125}I]-labelled fibrinogen (see *Methods*). As illustrated in Figure 13, pre-incubation with BSA caused marked inhibition of fibrinogen association with the neutrophil. This suggested that fibrinogen and BSA might compete for the same binding site(s) on the neutrophil surface.

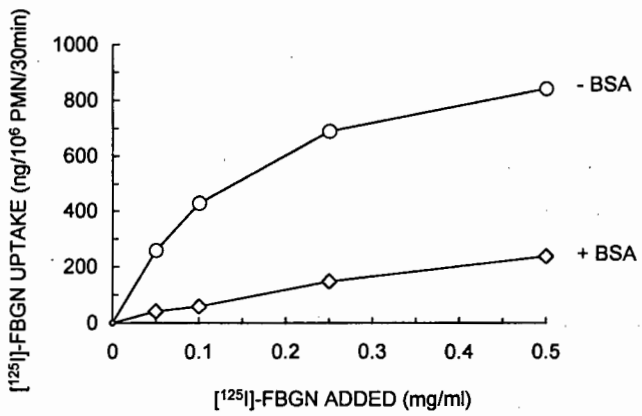


Figure 13: The effect of BSA on the association of [^{125}I]-labelled fibrinogen with PMN. *[^{125}I]-labelled fibrinogen (0-500μg/ml) was incubated (37°C, 30 minutes) with PMA stimulated PMN (5 x 10^6/ml) in the presence and absence of BSA (2mg/ml). Association of [^{125}I]-labelled fibrinogen was measured and expressed as ng per 10^6 PMN. Each data point represents the mean of duplicates. An additional experiment gave a similar result (see appendix 3, page 233).*

Discussion

This study aimed to investigate the association of neutrophil derived FDP with β_2-integrin receptors, as suggested by the results of earlier studies.[2] The present study therefore employed similar experimental conditions to those used in earlier studies.[2]

Immunoelectron microscopy revealed neutrophil associated FDP to be located within electron lucent vesicles suggesting that fibrinogen was being internalised by the neutrophil. Whilst the possibility that these "vesicles" might be external to the cell (i.e. plasma membrane invaginations, viewed in cross-section, see figure 14) was considered, several factors suggested that this was unlikely.

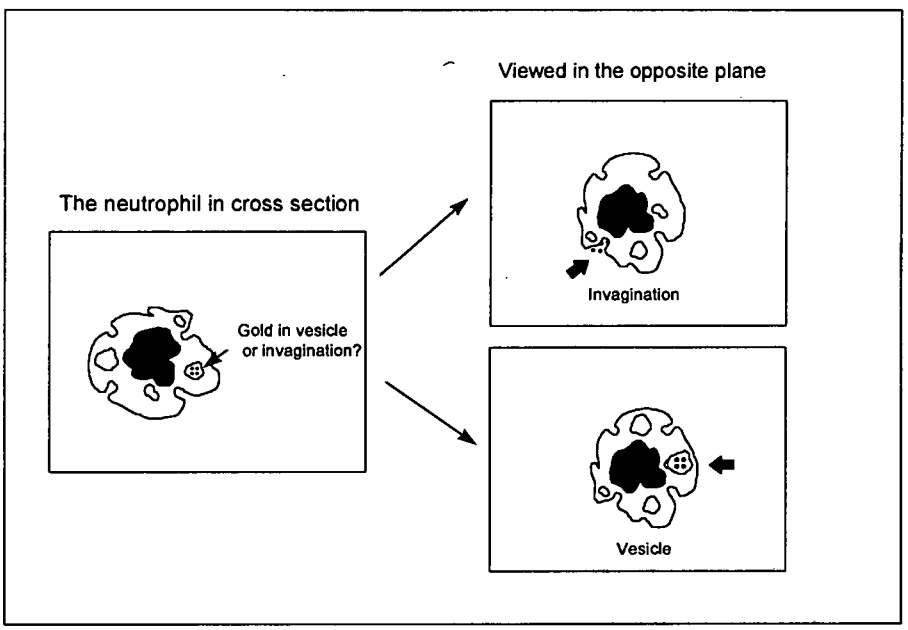


Figure 14: *It is possible that gold label which appears to be intracellular in cross-section, may, in fact, be extracellular if it is present in an invagination. This is illustrated in the figure above which shows the two possible locations of gold label which appears to be intracellular. This would become apparent if sections were cut in the opposite plane.*

- Firstly, if labelled structures were invaginations rather than vesicles, then external labelling of these structures should have been observed in invaginations viewed in the opposite plane (figure 14, top right). Such labelling was not observed.
- Secondly, if FDP were binding extracellularly to integrin receptors, their cell surface distribution should resemble that of CD11b/CD18 (one of its putative receptors) in activated neutrophils (figure 2b).* This was not observed with FDP.
- Thirdly, the pattern of intracellular labelling was very similar to that previously described for neutrophils internalising gold-conjugated BSA by fluid phase pinocytosis.[13]

Thus, it was likely that gold labelled vesicles represented intracellular FDP. This is further suggested by the fact that the conditions under which these experiments were performed were stimulatory for neutrophil pinocytosis. It has been reported that activation of neutrophils with phorbol ester (PMA)[24,25] or chemotactic peptide (FMLP)[13] results in a rapid increase in the rate of ligand independent membrane internalisation, and that phorbol esters, in particular, are extremely potent activators of this process.[25] Indeed, in the present study, PMA stimulated neutrophils (irrespective of whether fibrinogen was present) were noted to be highly vacuolated in comparison to non-stimulated cells. Under conditions such as these, surface bound ligand is often rapidly internalised, and as a result, studies investigating ligand binding to cell surface receptors, frequently employ conditions under which the endocytic apparatus is inhibited. This is usually accomplished by using cells that are chilled (0-4°C), [16,17,19] or the use of metabolic inhibitors such as sodium fluoride or sodium azide.[26,27] However, because the present study aimed to further characterise observations made in previous studies,[2] initial experiments were performed under the same conditions (i.e. PMA stimulation at 37°C) as those used previously.

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* Although expression of CD11b/CD18 was measured in FMLP stimulated rather that PMA stimulated neutrophils, both agents are known to have a similar effect on the cell surface expression of integrin receptors.[40]

The mechanism of fibrinogen internalisation

The next phase of this study aimed to determine the mechanism by which FDP accumulated intracellularly.

The observation that FDP accumulated intracellularly in the absence of significant binding to the neutrophil surface, suggested initially that uptake might occur via fluid phase pinocytosis. If this were the case, then it was possible that the previously documented neutrophil associated FDP[2] might reflect the uptake of *intact* fibrinogen which was subsequently degraded *intracellularly*. The uptake of fibrinogen was thus compared to that of BSA, a well described marker of fluid phase pinocytosis.

BSA (unlabelled[10] or tagged with fluorescein,[11] colloidal gold,[12-14] or [^{125}I][15,16]) has been widely used as a marker of non-specific fluid phase pinocytosis. Activated neutrophils have been shown to internalise large quantities of gold conjugated-BSA whilst showing minimal binding of this ligand to the neutrophil surface.[12,13] In addition, BSA has been used, both in monocytes[16] and platelets,[28,29] as a non-specific control when investigating uptake of fibrinogen via integrin receptors. A comparison of the uptake of fibrinogen and BSA thus appeared to be a relatively simple and convenient approach in determining the specificity of fibrinogen uptake by the neutrophil. These two proteins were found to gain access to the same intracellular compartments, and neither ligand accumulated on the neutrophil surface. Further studies, using radiolabelled fibrinogen and BSA, revealed these two proteins to be taken up in comparable quantities, and a large proportion of internalised fibrinogen was subsequently shown to be released into the extracellular medium in a similar manner to that reported for other molecules internalised by pinocytosing neutrophils.[14] These findings initially led to the suggestion that fibrinogen uptake was occurring predominantly via fluid phase pinocytosis. However, fluid phase pinocytosis is characterised by uptake which varies linearly with solute concentration over a relatively large (mg/ml) range.[17] As the dose response curves for the uptake of both fibrinogen and BSA are not linear, a proportion of uptake may be facilitated by saturable binding at the neutrophil surface. Divalent cations may indeed enhance the

uptake of these proteins. Consistent with earlier reports,[2] initial experiments had found the uptake of [^{125}I]-labelled fibrinogen to be similar in PBS (calcium free) and HBSS (which contains 1mM $CaCl_2$ and $MgCl_2$) suggesting that uptake was not dependent on divalent cations. However, following the observation that the pH of HBSS was not stable under the experimental conditions employed, assays were repeated in HEPES buffered HBSS. This resulted in a marked increase in the association of both fibrinogen and BSA with the neutrophil.

Thus the internalisation of fibrinogen and BSA might to some extent be preceded by binding to the neutrophil surface, particularly in the presence of divalent cations. To investigate this possibility, binding experiments (in HEPES buffered HBSS) were performed on ice to prevent internalisation of surface bound ligand. Whilst association of [^{125}I]-labelled fibrinogen and BSA was markedly diminished under these conditions, it was not abolished, suggesting that a degree of surface binding may occur. It thus appeared that binding of fibrinogen and BSA to the neutrophil surface might to some extent facilitate internalisation. The prior detection, by immunoelectron microscopy, of intracellular but not surface associated fibrinogen might be explained as follows:

- Immunoelectron microscopy experiments were performed at 37°C. At this temperature, ligands bound to the cell surface are rapidly internalised,[17,19] particularly in cells in which the pinocytic pathway is activated (such as those stimulated with PMA). Thus, the association of fibrinogen/FDP with the neutrophil surface may be transient due to its rapid internalisation, and therefore not detected under these conditions. In addition, at this temperature significant degradation of fibrinogen occurs due to release of proteases from PMA stimulated neutrophils which is enhanced in the presence of fibrinogen.[2] This process may rapidly and efficiently clear the surface of any fibrinogen. Such proteolysis has previously been reported to facilitate neutrophil detachment from fibrinogen coated surfaces.[30]
- Fibrinogen was used at a relatively high concentration (1mg/ml) during the electron microscopy study. The concentration of a ligand has a major influence on the pathway by which it is

internalised.[14] At low concentrations *receptor-mediated* uptake is favoured, whilst at higher concentrations internalisation via *fluid phase pinocytosis* also occurs.[14] This has been well documented for the chemotactic peptide fNNLP* (see table 1 and figure 15).[14] It is therefore possible that, at the fibrinogen concentrations used during electron microscopy studies, a significant proportion of fibrinogen/FDP is internalised via fluid phase pinocytosis without any prior contact with the neutrophil surface.

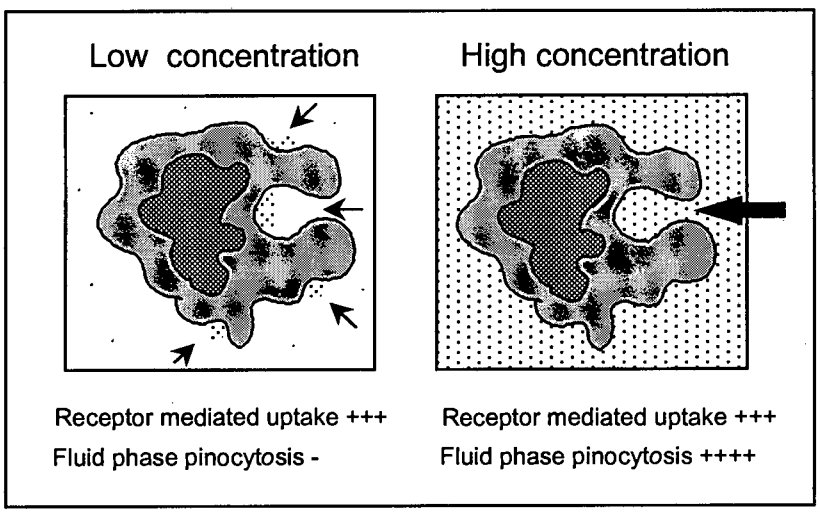


Figure 15: The effect of ligand concentration on receptor mediated endocytosis versus fluid phase pinocytosis in PMA stimulated neutrophils. *Receptor mediated endocytosis involves the selective concentration of a ligand at the cell surface. Thus, even at low concentrations of ligand, there is sufficient concentration at the cell surface to allow for uptake of significant quantities. Due to the low concentration of ligand in the surrounding medium, minimal uptake occurs via the fluid internalised during pinocytosis. As the concentration of ligand in the surrounding medium is increased, so the amount of ligand internalised in each fluid droplet during pinocytosis increases and constitutes an increasing proportion of total uptake.*

* N-formylnorleucylleucylphenylalanine

[^{3}H] FNNLP concentration	*Percentage of total uptake which is receptor mediated*
2×10^{-5} M	*<1*
2×10^{-6} M	*5*
2×10^{-7} M	*40*
2×10^{-8} M	*80*
2×10^{-9} M	*>90*

Table 1. *The influence of [^{3}H]-N-formylnorleucylleucylphenylalanine (FNNLP) concentration on its pathway of uptake by neutrophils at 37°C (adapted from reference 14).*

- In retrospect, the inclusion of BSA in the washing buffer (see *Methods*) may also have contributed to the failure of immunoelectron microscopy to demonstrate surface associated fibrinogen/FDP. As later experiments suggested that BSA might compete with fibrinogen for binding sites on the neutrophil surface (see below), it was possible that these washing steps might have removed any fibrinogen/FDP associated with the neutrophil surface.

As fibrinogen and BSA displayed similar patterns of uptake under all conditions employed during this study, it was possible that these proteins were taken up via a similar process. To investigate this possibility, competition studies were performed testing the ability of BSA to inhibit [^{125}I]-labelled fibrinogen association with the neutrophil. For these experiments fibrinogen was used at relatively low concentrations (0.05–0.5 mg/ml) which favour uptake via a surface mediated process. A marked decrease in the association of [^{125}I]-labelled fibrinogen with the neutrophil was noted in the presence of BSA suggesting that these proteins may compete for limited binding sites on the neutrophil surface. Since BSA is frequently included in incubation- and washing- buffers to block "non-specific binding", its ability to inhibit fibrinogen association with the neutrophil brings into question the specificity of this association.

The possibility that BSA inhibited fibrinogen binding due to competition for β_2-integrin receptors was also considered. Neutrophils have, in fact, been reported to adhere via β_2-integrin receptors, to surfaces coated with BSA and human serum albumin (HSA).[31,32] However, neutrophils are also able to adhere (β_2-integrin dependently) to a wide variety of other protein substrates when immobilised on plastic surfaces, via what appear to be non-specific glue-like interactions.[33] Included in this group, are proteins as varied as catalase, transferrin, soybean trypsin inhibitor, casein and ovalbumin.[34] Whilst the soluble forms of these proteins are not considered ligands for β_2-integrin receptors, surface bound forms of these proteins are. It has been suggested that partial unfolding following binding to plastic surfaces exposes domains which are recognised by β_2-integrin receptors.[34]

In contrast to *surface adherent* albumin, there is no evidence to suggest that *soluble* albumin can bind to β_2-integrins.[34,35] Whilst the specific binding of denatured albumin to neutrophils has been reported, native albumin does not exhibit specific binding.[35] A comparison of the amino acid composition of albumin to that of extracellular matrix proteins (fibronectin, vitronectin, von Willebrand's factor and fibrinogen) suggests that its interaction with β_2-integrins is unlikely.[36] Finally, the inability of soluble albumin to inhibit neutrophil binding to ECM protein coated surfaces, further suggests that it does not bind specifically to integrin receptors.[36]

Soluble HSA has, however, been shown to bind to the sialoprotein, sialophorin (CD43) on the neutrophil surface,[36] and it is thus possible that BSA may bind to the neutrophil via a similar mechanism. Recently, rat neutrophils and murine macrophages have been shown to internalise gold conjugated BSA via coated pits and it was suggested that uptake may involve scavenger receptors.[37] However, in a similar study using human neutrophils, it was shown that whilst early endocytic invaginations and small peripheral vesicles containing BSA-gold included coated areas of the membrane, uptake did not occur via a saturable, specific receptor mediated process.[13] Moreover, BSA has been used as a control protein in studies investigating integrin mediated uptake of fibrinogen in platelets[28,29] and monocytes.[16]

Interestingly, during a recent study investigating pinocytosis in rat hepatocytes,[38] it was noted that binding of BSA to chilled hepatocytes was considerably higher when BSA was directly labelled with [^{125}I]Na than when it was conjugated to tyramine cellobiose via its lysine residues. It was suggested that this may be due to non-specific adsorption of [^{125}I]-labelled BSA to the plasma membrane, and that conjugation of albumin to tyramine cellobiose may, in some way, alter the charge or hydrophobicity of the molecule, reducing its affinity for the plasma membrane.[38] It is possible that similar charge related or hydrophobic interactions may have accounted for the binding of [^{125}I]-labelled BSA to the neutrophil observed during the present study.

In contrast to BSA, fibrinogen is a recognised ligand for β_2-integrin receptors,[39-46] and the β_3-integrin receptor, leukocyte response integrin (LRI).[47,48] Most studies have characterised the binding of β_2-integrin receptors to *surface adherent* fibrinogen.[39,40,43-45] Although *soluble fibrinogen* has been reported to associate specifically with CD11b/CD18 on neutrophils and monocytes,[41,42,46] it has been suggested that the affinity of β_2-integrin receptors for *soluble fibrinogen* is very low.[39,43] Conformational changes in fibrinogen, such as those which occur following binding to glass or plastic surfaces, may be required to unmask integrin recognition sites.[42] It is thus thought that circulating monomeric fibrinogen will not bind to integrin receptors to a significant extent, and that clotted fibrinogen, or fibrinogen coated surfaces (in which the carboxyl terminus of the gamma chain is "multivalent") is the biologically relevant substrate for these receptors.[39,43] In the context of adhesive interactions between cells and the extracellular matrix, these multivalent, low affinity interactions sum to yield the biologically important phenomenon of adhesion.[39]

A recent study reporting the interaction of soluble FDP with β_2-integrin receptors on PMA stimulated neutrophils was the stimulus for the present study.[2] This report was based on three sets of observations:

- Firstly, when PMA stimulated neutrophils were incubated with [^{125}I]-labelled fibrinogen, washed, and then solubilised prior to analysis by SDS-PAGE, only [^{125}I]-labelled fibrinogen products of less than 30 kDa in size were detected on autoradiographs.[2]
- Secondly, the serine protease inhibitor AEBSF, which inhibits neutrophil mediated fibrinogen degradation, markedly inhibited the association of [^{125}I]-labelled fibrinogen (product) with the neutrophil under these conditions.*
- Thirdly, monoclonal antibodies directed against the CD11a, CD11b, CD11c and CD18 subunits of β_2-integrin receptors partially inhibited the association of these [^{125}I]-labelled products with neutrophils following incubation with [^{125}I]-labelled fibrinogen (see Table 2).[2]

Percentage inhibition of [^{125}I]-labelled fibrinogen association with neutrophils

	Source of antibody	
Monoclonal antibody	Serotec	Sanbio
No antibody	0	0
Anti-CD11a	40	31
Anti-CD11b	-	35
Anti-CD11c	30	45
Anti-CD18	10	10
OKM1 (anti-CD11b)	-	22
OKM10 (anti-CD11b)	-	18
All of the above	-	60
Anti-CD41a	-	0
Anti-CD16	-	25

Table 2. *The reported effect of monoclonal antibodies against various neutrophil receptors on the association of [^{125}I]-labelled fibrinogen (products) with PMA stimulated neutrophils (adapted from reference 2). [^{125}I]-labelled fibrinogen (1mg/ml) was incubated (37°C, 15 minutes) with PMA stimulated neutrophils in the presence and absence of monoclonal antibodies to various neutrophil receptors.*[2]

* AEBSF has recently been reported to react rapidly and non-specifically with many proteins to such an extent that a blocking reagent has been specifically designed to address this problem.[49] It is thus possible that the inhibitory effect of AEBSF on the neutrophil association with [^{125}I]-fibrinogen (products) might result from its binding to membrane proteins rather than from its inhibition of fibrinogen peptide formation.

Whilst these findings suggest a role for integrin receptors in the uptake of fibrinogen/FDP under these experimental conditions, several factors suggest that the inhibition achieved by these antibodies should be interpreted cautiously.

- Antibodies directed against the shared β-subunit of the β_2-integrin receptors, CD18, were relatively ineffective in blocking the association of [^{125}I]-labelled fibrinogen products with neutrophils (10% inhibition).[2] As it is well known that both the CD11 and CD18 subunits are required for ligand recognition by β_2-integrins,[50] the relatively minor inhibition exerted by anti-CD18 antibodies brings into question the mechanisms by which antibodies to the CD11 subunits exerted their inhibitory effects.*
- Neither CD11a/CD18 (which binds to ICAM-1 and -2 on endothelial cells) nor the IgG Fc receptor, CD16, are known receptors for fibrinogen.[39,51] However, antibodies directed against these receptors caused similar levels of inhibition to those directed against the α-subunits of the integrin receptors CD11b/CD18 and CD11c/CD18 (see table 1). This raises questions over the specificity with which the latter antibodies exerted their effects.
- It is likely that fluid phase pinocytosis contributed significantly to overall association of [^{125}I]-labelled fibrinogen products with the neutrophil under the conditions employed in these experiments. The pinocytic apparatus is highly activated in neutrophils stimulated with PMA at 37°C,[24,25] and the high concentrations of fibrinogen used (1mg/ml) favour uptake via fluid phase pinocytosis (see explanation page 187). Such uptake may well have accounted for the large proportion of uptake that could not be inhibited in the presence of anti-integrin antibodies.

From data acquired during the present study, it is unlikely that the reported association of [^{125}I]-labelled FDP (M_r<30kDa) with neutrophils reflects only selective binding of *FDP* to the neutrophil[2]. Such association is more likely the result of uptake of both intact and

*It is assumed that the two anti-CD18 antibodies used in this study were "blocking antibodies". It appears that the *Serotec* anti-CD18 antibody, at least, has been used as a blocking antibody in a previous study.[52]

degraded forms of fibrinogen. Intracellular degradation then reduces the internalised proteins to products of less than 30 kDa size. This is suggested by the immunolocalisation of fibrinogen (products) within intracellular vesicles and by their rapid release as FDP with apparent molecular mass values similar to those previously reported for neutrophil associated FDP.[2] Moreover, binding studies (performed at 0°C) suggest that neutrophils do not show a preference for binding to extensively degraded fibrinogen compared to intact fibrinogen.
Such a view is not consistent with the reported inhibitory effect of low M_r FDP on neutrophil adherence to immobilised fibrinogen.[2] However, a review of the experimental data from that study suggests that a lack of adequate controls may well have accounted for the observations leading to that conclusion (personal communication).

Summary and conclusions

It has previously been hypothesised that fibrinogen proteolysis by the neutrophil membrane generates low molecular weight FDP which associate specifically with β_2-integrin receptors, and can block adhesive interactions with the extracellular matrix (see figure 1, page 161).[2]

The present study, which sought morphological evidence to support this hypothesis, has found evidence to challenge it. On the basis of observations made during this study, it is postulated that FDP becomes associated with the neutrophil via the following mechanism (see figure 16).

1. Neutrophils stimulated with PMA at 37°C, undergo ligand independent internalisation of their plasma membranes. Under these conditions fibrinogen and FDP* in the surrounding medium

* Under the experimental conditions employed both intact and degraded fibrinogen are present in the surrounding medium.[1] This is due to neutrophil mediated fibrinogenolysis which yields both high and low molecular weight fibrinogen degradation products.

are internalised both by fluid phase pinocytosis and via association with the plasma membrane. The presence of divalent cations appears to favour the latter, whilst the former is enhanced at higher concentrations of ligand. The neutrophil does not appear to discriminate between degraded and non-degraded forms of fibrinogen. Data from previous studies suggests that β_2-integrin receptors may, to some extent, be involved in this process. However the observation, during the present study, that BSA can inhibit fibrinogen association with the neutrophil, suggests adherence to cell surface molecules other than β_2-integrin receptors should also be considered.

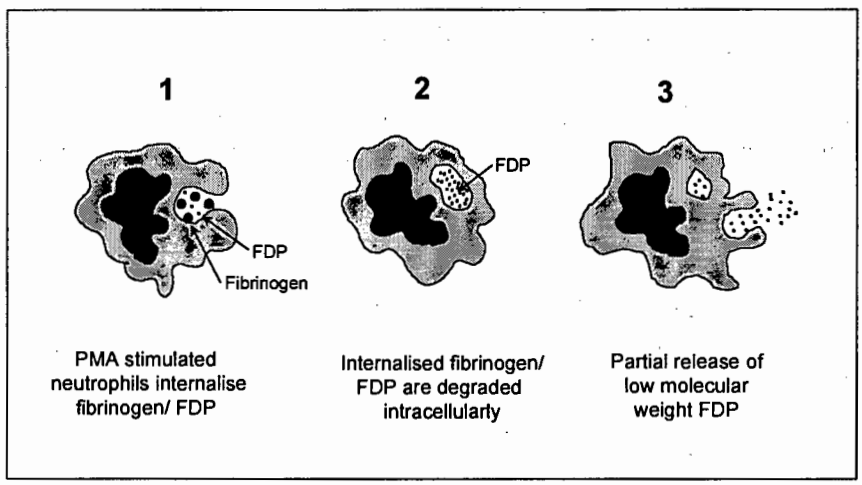


Figure 16: *The proposed mechanism whereby FDP become neutrophil associated during incubation of PMA stimulated neutrophils with fibrinogen.*

2. Internalised fibrinogen (products) are rapidly degraded intracellularly into low molecular weight FDP. This degradation may be mediated by HNE and cathepsin G which are known to be expressed on the neutrophil plasma membrane (and therefore on membranes of pinocytic vesicles) under these conditions.

3. A proportion of these products are released back into the surrounding medium. Those that remain cell associated most likely account for the low molecular weight fibrinogen peptides previously reported in association with the neutrophil.[2] It is

therefore unlikely that these products modulate neutrophil β_2-integrin dependent adhesive interactions as has previously been suggested.[2]

Finally, despite the widespread use of BSA as a marker of fluid phase pinocytosis, this study suggests that [^{125}I]-BSA, due to its propensity to bind to the neutrophil surface, is not a suitable marker of fluid phase pinocytosis in this setting. As a result the use of BSA did not allow for the elucidation of the pathway of fibrinogen uptake as was originally intended.

References

1. Kelly SL, Adams SA, Robson SC, Kirsch RE, Shephard EG. (1994) *Biochem. J.* **298**, 689.
2. Kelly SL. (1995) A neutral protease of the neutrophil surface: its role in the proteolysis of C-reactive protein and fibrinogen - PhD Thesis.
3. Tokayasu KT. (1980) *Histochem J.* **12**, 281.
4. Tokayasu KT. (1986) *J. Microscopy.* **143**, 139.
5. Cameron CHS, Toner PG. *Electron microscopic immunocytochemistry in histopathology.* In *Electron Microscopic Immunocytochemistry.* Eds. Polak JM, Priestly JV. (1992) Oxford University Press, Oxford. pp 11-35.
6. Komuves. (1992) *J. Histochem. Cytochem.* **40**, 1637.
7. Yun. (1992) *Pathology.* **24**, 91.
8. Bertolatus (1990) *J. Histochem. Cytochem.* **38**, 377.
9. Odeyale (1988) *J. Histochem. Cytochem.* **36**, 1131.
10. Apple RJ, Domen PL, Muckerheide A, Michael JG. (1988) *J. Immunol.* **140**, 3290.
11. Stein CA, Tonkinson JL, Zhang LM, Yakubov L, Gervasoni J. (1993) *Biochemistry.* **32**, 4855.
12. Berger M, Wetzler E, Welter E, Turner JR, Tartakoff AM. (1991) *Proc. Natl. Acad. Sci. USA.* **88**, 3019.
13. Berger M, Wetzler E, August JT, Tartakoff AM. (1994) *J. Clin. Invest.* **94**, 1113.
14. Daukas G, Lauffenburger DA, Zigmond S. (1983) *J. Cell Biol.* **96**, 1642.
15. Storrie B, Maurey KM. (1981) *J. Cell Sci.* **50**, 135.
16. Simon DI, Ezratty AM, Francis SA, Rennke H, Loscalzo J. (1993) *Blood.* **82**, 2414.
17. Steinman RM, Mellman IS, Muller WA, Cohn ZA. (1983) *J. Cell Biol.* **96**, 1.
18. Steinman RM, Brodie SE, Cohn ZA. (1978) *J. Cell Biol.* **68**, 665.
19. Stahl P, Schlesinger PH, Sigardson E, Rodman JS, Lee Y.C. (1980) *Cell.* **19**, 207.

20. Melman I. (1982) *Ciba Found. Symp.* **92**, 35.
21. Adams CJ, Maurey KM, Storrie B. (1982) *J. Cell Biol.* **92**, 632.
22. Besterman JM, Airhart JA, Woodworth RC, Low B. (1981) *J. Cell Biol.* **91**, 716.
23. Tietze C, Schlesinger P, Stahl P. (1982) *J. Cell Biol.* **92**, 417.
24. Robinson JN, Badwey JA, Karnovsky ML, Karnovsky MJ. (1987) *J. Cell Biol.* **105**, 417.
25. Keller HU. (1990) *J. Cell. Physiol.* **145**, 465.
26. Steinman RM, Cohn ZA. (1972) *J. Cell Biol.* **55**, 186.
27. Sherman LA, Lee J. *J. Exp. Med.* (1977) **145**, 76.
28. Handagama PJ, Scarborough RM, Shuman JA, Bainton DF. (1993) *Blood.* **82**, 135.
29. Handagama PJ, Amrani DL, Shuman MA. (1995) *Blood.* **85**, 1790.
30. Cai T-Q, Wright SD. (1996) *J. Exp. Med.* **184**, 1213.
31. Stocks SC, Kerr MA, Haslett C, Dransfield S. (1995). *J. Leuk. Biol.* **58**, 40.
32. Simon SI, Burns AR, Taylor AD, Gopalan PK, Lynam EB, Sklar LA, Wayne Smith C. (1995), *J. Immunol.* **155**, 1502.
33. Graham IL, Brown EJ. (1991) *J. Immunol.* **146**, 685.
34. Davis GE (1992) *Exp. Cell Res.* **200**, 242.
35. Wilkinson PC, Allan RB. (1978) *Mol. Cell. Biochem.* **20**, 25.
36. Nathan C, Xie Q, Halbwachs-Mecarelli L, Wen Jin W. (1993) *J. Cell Biol.* **122**, 243.
37. Vannier-Santos MA, de Souza SJ, Brentani RR. De Souza W. (1996) *Biochem. Biophys. Res. Comm.* **221**, 837.
38. Stromhaug PE, Berg TO, Gjoen T, Seglen PO. (1997) *Eur. J. Cell Biol.* **73**, 28.
39. Wright SD, Weitz JI, Huang AJ, Levin SM, Silverstein SC, Loike JD. (1988) *Proc. Natl. Acad. Sci. USA.* **85**, 7734.
40. Diamond MS, Garcia-Aguilar J, Bickford JK, Corbi AL, Springer TA. (1993) *J. Cell Biol.* **120**, 1031.
41. Altieri DC, Plescia J, Plow EF. (1993) *J. Biol. Chem.* **268**, 1847.
42. Altieri DC, Bader R, Mannuci DM, Edgington TS. (1988) *J. Cell Biol.* **107**, 1893.
43. Loike JD, Sodeik S, Cao L, Leucona, Weitz J, Detmers PA, Wright SD, Silverstein SC. (1991) *Proc. Natl. Acad. Sci. USA.* **88**, 1044.
44. Fuortes M, Jin WW, Nathan C. (1993) *J. Cell Biol.* **120**, 777.
45. Berton G, Fumagalli L, Laudanna C, Sorio C. (1994) *J. Cell Biol.* **126**, 1111.
46. Ugarova TP, Solovjov DA, Zhang L, Loukinov DI, Yee VC, Medved LV, Plow EF (1998) *J. Cell Biol.* **273**, 22519.
47. Gresham HD, Goodwin JL, Allen PN, Anderson DC, Brown EJ. (1989) *J. Cell Biol.* **108**, 1935.
48. Gresham HD, Adams SP, Brown EJ. (1992) *J. Biol. Chem.* **267**, 13895.
49. Boehringer Mannheim product specifications: Pefablock SC® Plus.
50. Loftus JC, Smith JW, Ginsberg MH. (1994) *J. Biol. Chem.* **269**, 25235.
51. Luscinskas FW, Cybulsky MI, Kiely JM, Peckin CS, Davis VM, Gimbrone MA. (1991) *J. Immunol.* **146**, 1617.
52. Higazi AA, Barghouti II, Ayesh SK, Mayer M, Matzner Y. (1994) *Inflammation.* **18**, 525.

Chapter Seven

Neutrophil mediated clot lysis

Once a fibrin clot has achieved haemostasis it needs to be dismantled - failure to do so will result in life-threatening thromboembolic events. Whilst the plasminolytic system serves as the primary pathway for fibrin dissolution, the neutrophil provides an alternative pathway and recent evidence suggests that neutrophil proteases may act synergistically with plasmin to aid clot lysis[1] (see chapter 3, pages 81-85).

Neutrophils have long been known to degrade fibrin via their *azurophil granule* proteases, HNE and cathepsin G.[2,3] More recently, the neutrophil *membrane* has been reported to be an important source of fibrinolytic activity.[4,7] This activity was ascribed to a 600 kDa protease which also degrades fibrinogen[5] and CRP[6] (see chapter 4). In studies using intact neutrophils, this fibrinolytic activity was shown to be constitutively present on the neutrophil membrane and was only marginally upregulated following stimulation with low dose PMA.[7]

Whilst the fibrinolysis observed during these studies appeared to be due to the neutrophil membrane,[4,7] it was possible that *intracellular digestion* might also contribute to this process. This possibility was entertained during the present study for several reasons:

- Firstly, the ability of the neutrophil to internalise and digest material in the pericellular environment was highlighted by the findings presented in the previous chapter.
- Secondly, neutrophil internalisation of partially digested fibrin particles had been shown, under certain conditions, to contribute significantly to the process of fibrin dissolution.[8,9]
- Finally, an immunostaining technique capable of detecting fibrinogen *degradation products* had recently been established (see previous chapter). This technique had the potential to detect

intracellularly degraded fibrin which is often not identifiable morphologically following degradation. [8]

The present study aimed to determine whether the neutrophil mediated fibrinolysis reported previously was exclusively due to the neutrophil membrane, or whether intracellular degradation also contributed to this process.

Several observations made during the course of this study raised questions over the nature of the membrane associated proteolytic activity - in particular the extent to which it was constitutively present on the membrane of resting neutrophils, as well as its origin. Additional studies were thus undertaken to further characterise this membrane associated fibrinolytic activity.

Methods

Chemicals and Reagents

The sources of chemicals and reagents used in this study are listed in *Appendix 1*, page 225-226.

Neutrophil isolation and fibrinogen purification & iodination

These procedures were performed as described previously (see chapter 4, *Methods* page 107). Purified neutrophils were resuspended in HEPES buffered HBSS, and kept on ice until the beginning of experimental procedures.

Incorporation of neutrophils into fibrin clots

Neutrophils were incorporated into fibrin matrices using adaptations of previously described procedures.[4,7] Neutrophils (20 x 10^6/ml) in HEPES buffered HBSS) were warmed (37°C, 10 minutes), and following the addition of bovine thrombin (0.25units/ml, final concentration), were mixed with fibrinogen (1mg/ml, final concentration) in a final reaction volume of 200µl. Where neutrophils were stimulated with PMA (10ng/ml, final concentration), this was added immediately prior to the addition of fibrinogen. The reaction was allowed to proceed at 37°C for various durations of time. At selected time points samples were removed from the waterbath, and fixed (3.5% paraformaldehyde and 0.2% glutaraldehyde, 0.1M phosphate buffer, pH=7.4) on ice for 90 minutes.

Gelatin embedding

Following fixation, fibrin clots were rinsed and incubated with 0.05M glycine in PBS to quench free aldehydes, before embedding in gelatin. This involved the infiltration of fibrin clots with increasing

concentrations of gelatin (37°C, rotating end-on-end) according to the following protocol: 2% gelatin in PBS x 20 minutes; 6% gelatin in PBS x 20 minutes; 10% gelatin in PBS x 20 minutes. In order to avoid any fibrinolytic activity that might be reactivated following the removal of fixative, the serine protease inhibitor AEBSF (final concentration 1mM) was added to the 2% gelatin mixture. Following gelatin infiltration fibrin clots were gently removed, placed on a glass slide and cooled to 4°C, to solidify the gelatin. The clot was transferred into a tube containing fixative (same as above) and kept at 4°C overnight in order to crosslink the infiltrated gelatin. The next morning the gelatin infiltrated clots were removed from fixative, rinsed with PBS, and cut into small blocks (approximately $1mm^3$) with a scalpel blade.

Ultracryotomy and immunostaining

Blocks were sucrose infiltrated and cryosectioned, and sections immunostained (with antifibrinogen antibody) as described earlier. (see chapter 6 pages 164-166). Sections were then analysed by transmission electron microscopy.

Immunohistochemistry

For the preparation of sections for light microscopy, the same procedure was followed with the exception that the gelatin infiltrated clots were not cut into blocks, but instead embedded in paraffin prior to microtomy. Thin sections were dewaxed and pretreated with 0.3% hydrogen peroxide in methanol (30 minutes, room temperature) to quench endogenous peroxidase activity. Sections were incubated with normal swine serum (diluted 1:20 in PBS) for 15 minutes, prior to exposure (30 minutes, room temperature) to rabbit antihuman fibrinogen antibody (10µg/ml) in PBS. Slides were then washed with PBS and incubated (30 minutes, room temperature) with biotinylated swine antirabbit IgG diluted 1:250 in PBS. After washing with PBS slides were incubated for a further 30 minutes with horseradish peroxidase linked streptavadin (diluted 1:500 in PBS). Slides were then developed using the chromogenic substrate $3\text{-}3^1$ diamino-benzidine (0.5mg/ml) in the presence of 0.01% hydrogen peroxide.

Slides were then stained with Meyer's haematoxylin, dehydrated and mounted. Controls were included in which rabbit antihuman fibrinogen antibody was substituted with non-immune rabbit IgG.

Scanning electron microscopy

Following fixation clots were washed and then post-fixed in osmium tetroxide for two hours. Clots were then washed with distilled water and dehydrated through a graded series of ethanol concentrations before undergoing critical point drying. Clots were then sputter coated with gold palladium and analysed by scanning electron microscopy.

Neutrophil solubilisation of [^{125}I]-labelled fibrin clots

Neutrophils were incorporated into fibrin matrices as described above with the exceptions that neutrophils were used at a concentration of 10 x 10^6/ ml and [^{125}I]-labelled fibrinogen was used to generate the clots. At selected time points, tubes containing fibrin clots were placed on ice, the clots squeezed and the supernatant collected and microfuged (11000 x g for 1 minute) to remove cellular debris. A 50µl aliquot was then removed and its radioactivity counted, and used as a measure of clot solubilisation. Radioactivity not incorporated into fibrin formed from [^{125}I]-labelled fibrinogen (in the absence of cells) was used as the measure of baseline soluble radioactivity in the reaction.

Analysis of [^{125}I]-labelled fibrin degradation products

[^{125}I]-labelled fibrin clots were generated by incubating fibrinogen (1.25mg/ml) with thrombin (0.6units/ml) in a final volume of 100µl HBSS-HEPES at 37°C for 2 hours. Clots were squeezed and added to either conditioned medium or granule enzymes (prepared as described in chapter 5, page 128) used at various concentrations (see results) and the reaction was allowed to proceed at 37°C for 20 hours. The proteolytic products were analysed by SDS-PAGE (10%, reducing) and autoradiography.

Results

Neutrophil mediated fibrinolysis is predominantly extracellular

PMA-stimulated neutrophils usually achieved complete dissolution of fibrin clots within 60-90 minutes (as judged macroscopically), whereas non-stimulated neutrophils induced no discernible alterations in the macroscopic appearance of the clot over the same period of time (figure 1).

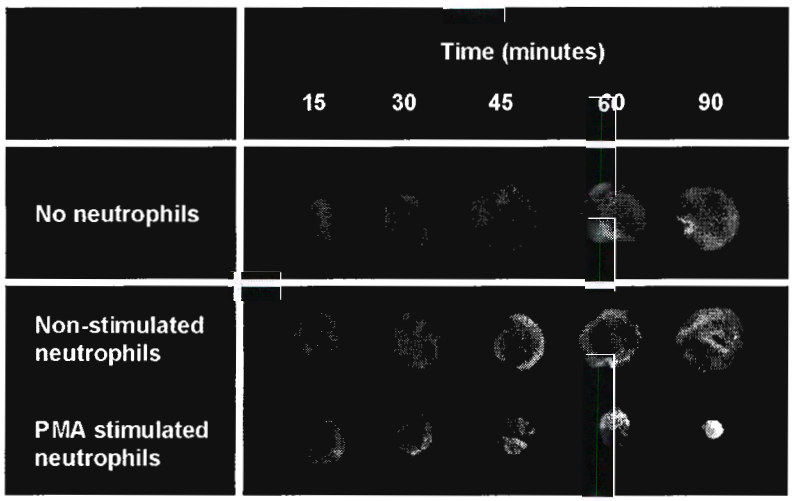


Figure 1: *Fibrin clots were generated in the absence of neutrophils or around non-stimulated or PMA (10ng/ml) stimulated neutrophils (20 x 10^6/ml) (see Methods). At 90 minutes, fibrin clots containing PMA stimulated neutrophils (right) showed near complete dissolution whilst those containing non-stimulated neutrophils (left) showed no decrease in size or density compared to clots formed in the absence of neutrophils.*

Immunoelectron microscopy revealed no evidence of fibrin internalisation by non-stimulated neutrophils over a time course of 0-90 minutes. A small percentage of PMA stimulated neutrophils (less than 5%) showed evidence of fibrin internalisation (figure 2 a-d). In a few cases, fibrin was detected within endocytic vesicles, mainly at later time points (30-90 minutes). Degraded fibrin, not identifiable

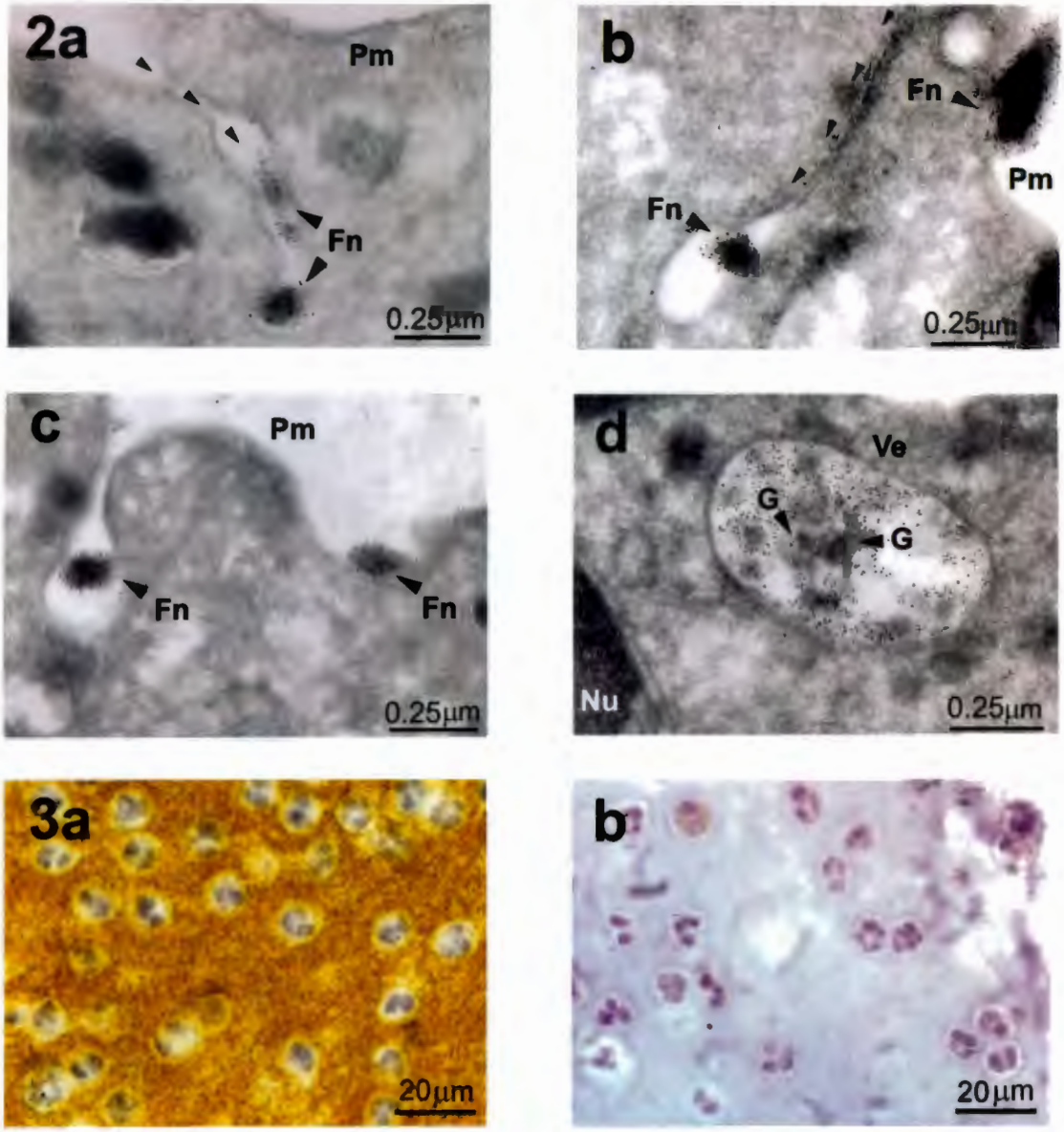


Figure 2: Transmission electron micrographs of PMA stimulated neutrophils showing evidence of fibrin internalisation. *PMA (10ng/ml) stimulated neutrophils (20 million/ml), incorporated within polymerising fibrin (1mg/ml) clots, were incubated for 30 minutes at 37 degrees C. Samples were fixed, cryosectioned and immunostained using rabbit antihuman fibrinogen antibody followed by 5nm-gold conjugated goat antirabbit IgG (see Methods). A small percentage (< 5%) of neutrophils showed evidence of fibrin internalisation with fibrin strands located within plasma membrane (Pm) invaginations (figures 2a-c) - arrows indicate the direction of the plasma membrane invaginations. Figure 2d shows the immunodetection of extensively degraded fibrin (no longer identifiable morphologically) within an intracellular vesicle containing cytoplasmic granules (G).*

Figure 3: Immunohistochemistry of PMA stimulated neutrophils within fibrin clots. *PMA stimulated neutrophils were incorporated within polymerising fibrin clots under the same conditions as described above. Sections were immunostained with rabbit antihuman fibrinogen antibody which was detected using the biotin-streptavadin-HRP system (see Methods). (a) Fibrin (stained brown) was detected extracellularly but not intracellularly. (b) A negative control, in which antifibrinogen antibody was substituted with non-immune rabbit IgG, confirmed the specificity of fibrin staining.*

morphologically, could be detected immunologically (figure 2d) confirming the ability of this procedure to detect degraded fibrin which was no longer identifiable morphologically. These findings were consistent over a series of 5 experiments in which at least 250 neutrophils were viewed, and were supported by immuno-histochemistry which showed a lack of cytoplasmic staining for fibrin (see figure 3a).

As minimal intracellular accumulation of fibrin was detected during the period in which complete fibrin dissolution was observed (macroscopically), it appears that neutrophil mediated fibrinolysis, under these conditions, is predominantly an extracellular event.

The effect of PMA on neutrophil mediated fibrinolysis

During this series of experiments, PMA stimulated neutrophils were noted to effect complete fibrin dissolution within 60-90 minutes, whilst over the same time period, non-stimulated neutrophils induced no noticeable changes in the size of the clot (figure 1). This suggested that the constitutive fibrinolytic activity of *non-stimulated* neutrophils was relatively low, and that neutrophil activation was required for this process. Previous studies had, however, reported only marginal upregulation of neutrophil mediated [^{125}I]-fibrinolysis following stimulation with PMA.[4,7] Further experiments were thus performed to investigate the effect of neutrophil activation on its capacity to degrade fibrin.

Quantification of [^{125}I]-labelled fibrin solubilisation

Solubilisation of [^{125}I]-labelled fibrin clots in which either non-stimulated or PMA stimulated neutrophils were incorporated was investigated as described previously[4,7] (figure 4). Control samples (fibrin clots without neutrophils) were included for each time point (figure 4a), and the soluble radioactivity in these samples was subtracted from experimental samples to estimate the rate of fibrinolysis (figure 4b). [^{125}I]-labelled fibrin solubilisation was found to be far faster for neutrophils stimulated with PMA than for

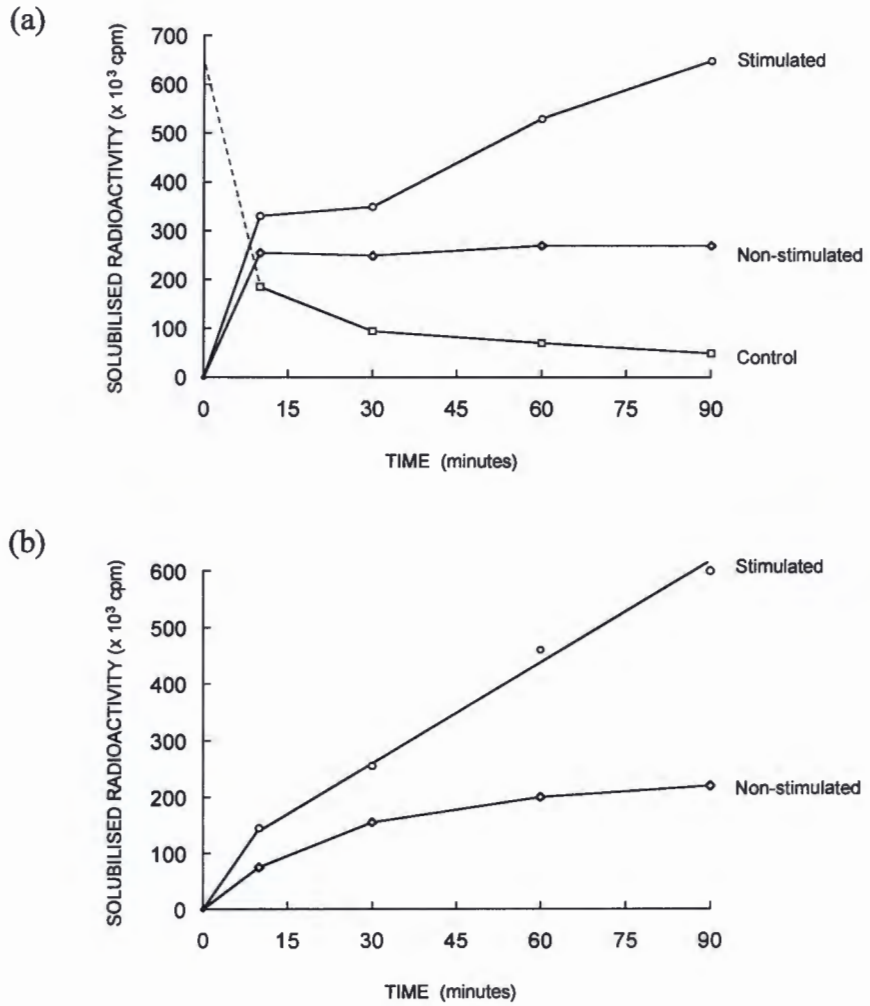


Figure 4: Comparison of [^{125}I]-labelled fibrin solubilisation by non-stimulated and PMA stimulated neutrophils. *(a) Non-stimulated or PMA stimulated neutrophils (10 x 10^6/ ml) were incorporated (37°C, 10-90 minutes) into 1mg/ml [^{125}I]-labelled fibrin clots. At selected time points solubilised radioactivity was measured and expressed as cpm released. The soluble radioactivity associated with cell free control clots was also measured at each time point. The progressive decrease in the soluble radioactivity seen in cell free control clots reflects fibrinogen polymerisation. (b) This figure shows the radioactivity released by stimulated and non-stimulated neutrophils experimental samples following the subtraction of radioactivity associated with the cell free control at each time point. Data points represent means of duplicates. Two additional experiments gave similar results (see appendix 3, pages 234-235).*

non-stimulated neutrophils. At 90 minutes PMA stimulated neutrophils achieved full solubilisation of the fibrin clot whereas non-stimulated neutrophils achieved less than 50% solubilisation.

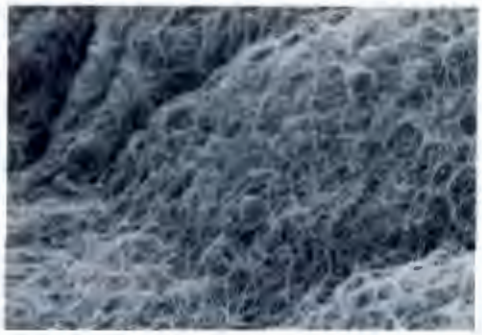
Scanning electron microscopy

Scanning electron microscopy revealed marked differences in the rate of fibrin solubilisation by PMA stimulated neutrophils compared to non-stimulated neutrophils. Non-stimulated neutrophils caused little appreciable change in the structure of the fibrin matrix over a period of 15 to 60 minutes (figure 5, right panel). These cells did not appear to undergo major morphological changes and remained rounded throughout the period of observation. In contrast, PMA stimulated neutrophils induced marked changes in the appearance of the fibrin matrix over the same time period (figure 5, left panel). At 15 minutes, the fibrin matrix appeared relatively dense with few visible neutrophils, but by 30 minutes the matrix had thinned substantially and more neutrophils had become exposed. The cells appeared far more concentrated due to collapse of the fibrin architecture, and many neutrophil aggregates were present. PMA stimulated cells underwent dramatic shape changes, exhibiting extensive pseudopod formation as well as vacuolation. By 60 minutes, hardly any fibrin was present in the vicinity of neutrophils and disintegration of the clot resulted in a highly concentrated mass of neutrophils, most of which were in aggregates. These findings suggest that neutrophil mediated fibrinolysis is markedly upregulated following stimulation with PMA.

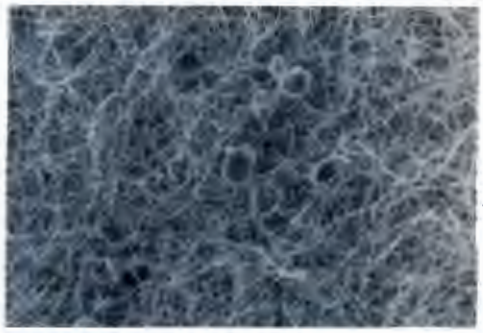
Figure 5 (Opposite): Scanning electron micrographs showing fibrin solubilisation by PMA stimulated and non-stimulated neutrophils. *PMA (10ng/ml) stimulated or non-stimulated neutrophils (20 x 10^6/ml) were incorporated within polymerising fibrin clots (1mg/ml) for 15-60 minutes at 37°C. Samples were then fixed and analysed by scanning electron microscopy. Clots containing PMA stimulated neutrophils are shown in the left hand panel and those containing resting neutrophils are shown on the right (Magnification x 5000). These samples are shown at higher magnification (x10 000) on the following page.*

PMA stimulated

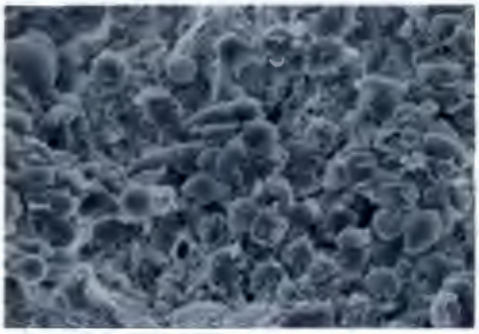
Non-stimulated



15 minutes

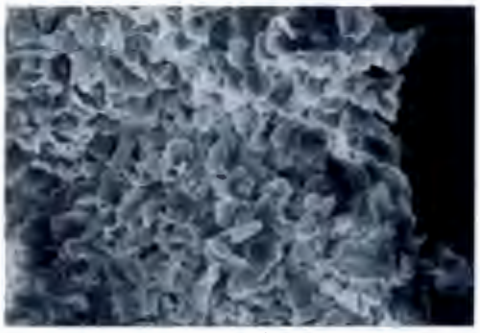


15 minutes

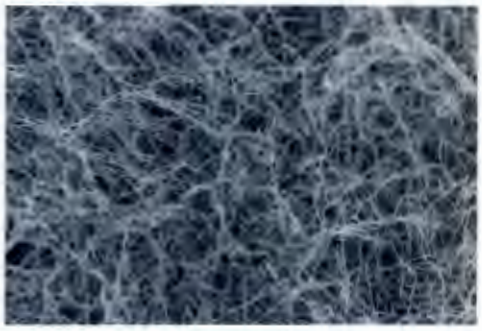


30 minutes

30 minutes



60 minutes



60 minutes

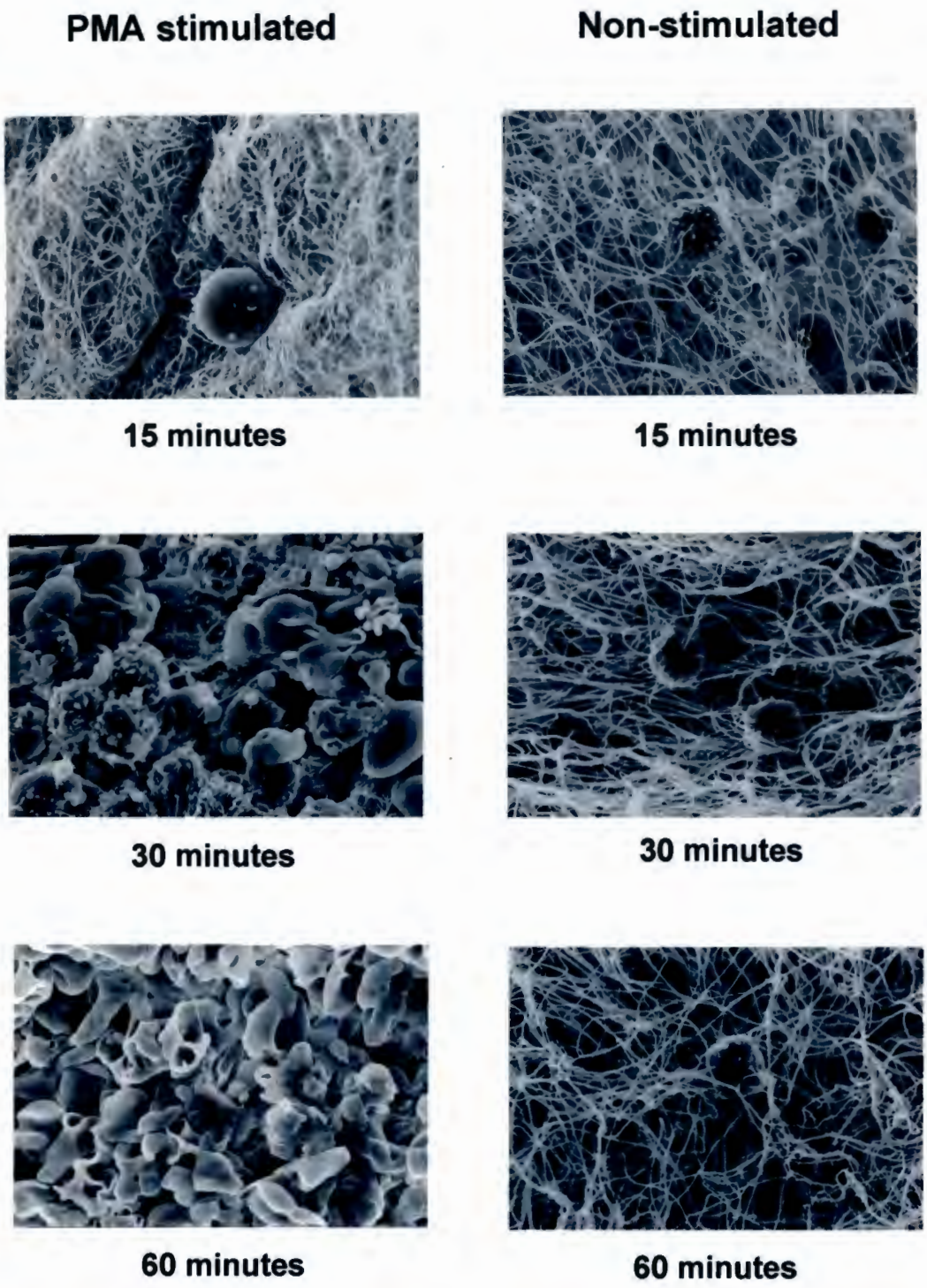


Figure 5. Scanning electron micrographs showing fibrin internalisation by PMA stimulated and non-stimulated neutrophils. *See previous page (Magnification x 10000).*

The role of granule proteases in fibrin dissolution

The possibility that PMA upregulated neutrophil mediated fibrinolysis by inducing release of azurophil granules was considered. Indeed, concurrent experiments (see chapters 4 and 5) revealed biochemical evidence to suggest that low dose PMA is capable of inducing azurophil granule release. Moreover, during the present study PMA stimulated neutrophils were noted to have a less granular cytoplasm than non-stimulated neutrophils and occasional granules were noted on the neutrophil surface (figure 6). It was possible, however, that these observations were due to processing steps during cryoultramicrotomy, which can result in extraction of cytoplasmic organelles. As a result, *resin embedded* sections of PMA stimulated- and non-stimulated neutrophils incorporated within fibrin matrices (37°C, 30 minutes) were used for a comparison of cytoplasmic granule content. This revealed the cytoplasm of non-stimulated neutrophils to be densely packed with granules whilst the majority of PMA stimulated neutrophils showed a decrease in granule content (figure 7).* The distribution of granules appeared to be altered in PMA stimulated neutrophils; there was a tendency for granules to redistribute towards the periphery of the cytoplasm with a relative depletion in the perinuclear area. These findings suggest that PMA is capable of inducing significant release of cytoplasmic granules under these conditions.

Analysis of fibrin degradation products: SDS-PAGE

To determine whether the release of neutrophil granules contributed to the fibrinolytic activity of the neutrophil membrane, membrane-

* Although attempts were made to quantify these differences, a quantitative comparison was precluded by a number of technical factors: Cell to cell comparisons could not be made because the diameters of cells differed depending upon which part of the cells were sectioned. Granule density per unit area was also not reliable due to distortions in the shape of PMA stimulated neutrophils as well as extensive vacuolation, which necessitated the selection of specific areas to examine, thereby introducing an element of subjectivity. As a result the comparison was made purely qualitatively. The differences between non-stimulated and stimulated neutrophils (figure 7) were clearly visible and were confirmed by an unbiased observer.

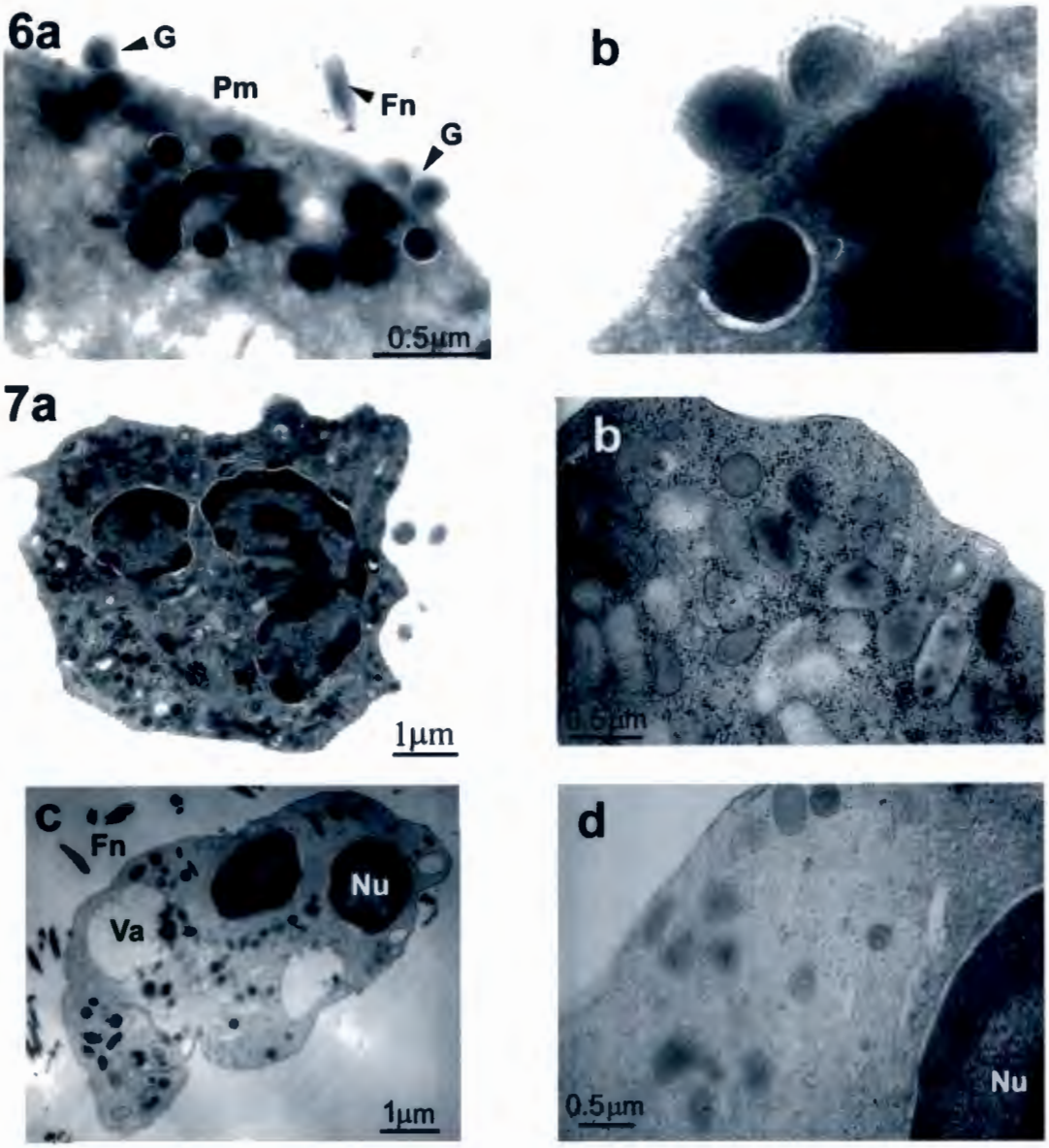


Figure 6: Transmission electron micrographs showing granules on the surface of a cryosectioned neutrophil. *(a) Cytoplasmic granules (G) were occassionally seen on the plasma membranes (Pm) of PMA stimulated neutrophils incorporated within fibrin (Fn) matrices during the experiments described in figure 2. (b) The same granules seen at higher magnification.*

Figure 7: Transmission electron micrographs showing the cytoplasmic granule content of PMA stimulated and non-stimulated neutrophils. *PMA (10ng/ml) stimulated and non-stimulated neutrophils (20 million/ml) were incorporated within fibrin matrices for 30 minutes at 37 degrees C. Samples were fixed, resin embedded and analysed by transmission electron microscopy. Representative micrographs show: (a) A non-stimulated neutrophil with densely packed cytoplasmic granules; (b) A higher magnification view of the cytoplasm of a non-stimulated neutrophil; (c) A PMA stimulated neutrophil with a lower density of cytoplasmic granules and vacuolation (Va) of the cytoplasm (Fn = fibrin, Nu = nucleus); (d) A higher magnification view of the cytoplasm of a PMA stimulated neutrophil.*

associated fibrinolytic activity was compared to that of granule proteases* with respect to the [^{125}I]-labelled degradation products that it generated. The conditioned medium of PMA (5ng/ml) stimulated neutrophils was used as a source of the membrane associated protease.[4,7] Degradation reactions were performed over a range of different protease concentrations and products were analysed by SDS-PAGE and autoradiography.

When sufficiently diluted, preparations of granule proteases generated identical appearing fibrin degradation products to those generated by the conditioned medium (figure 8). This suggests that the fibrinolytic activity of the conditioned medium (and therefore the neutrophil membrane) is due to proteases of the azurophil granule.

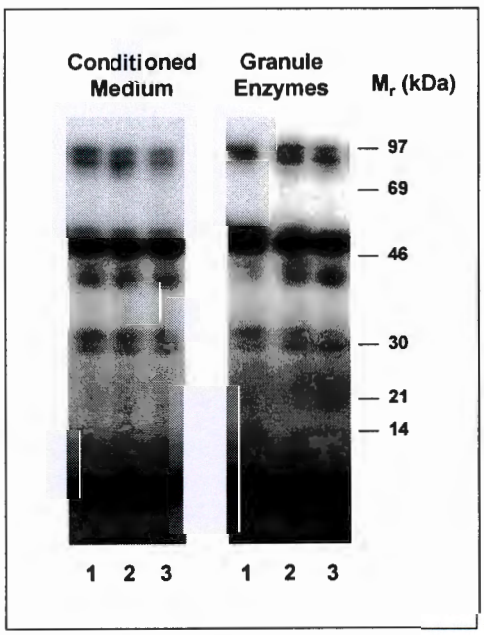


Figure 8: *A comparison of fibrin products generated by PMA stimulated neutrophil conditioned medium versus granule enzymes. [^{125}I]-labelled fibrin clots (125μg, 100μl) were incubated with either conditioned medium or granule enzymes (37°C, 20 hours). The proteolytic products generated were analysed by SDS-PAGE (10%, reducing) and autoradiography. Left panel lanes 1-3 show fibrin degradation products generated by conditioned medium from 3-, 3.5-, and 4 x 10^5 PMA stimulated neutrophils, whilst the right panel shows fibrin degradation products generated by granule enzymes (see Methods) from 1.6-, 2- and 2.4 x 10^5 neutrophils respectively.*

* Prepared as in Chapter 5, *Methods*, page 128.

Discussion

The initial purpose of this study was to determine the extent to which intracellular fibrin degradation contributed to neutrophil mediated fibrinolysis reported in earlier studies.[4,7]

Over the period in which complete fibrin dissolution was observed (macroscopically), immunoelectron microscopy revealed only a small percentage (<5%) of neutrophils to contain intracellular fibrin, suggesting that the neutrophil mediated fibrinolysis described in previous studies,[4,7] is predominantly an extracellular event.

Whilst other studies have reported neutrophil internalisation of fibrin,[8,9] these studies differed in experimental design from the present study. Riddle and Barnhart[8] studied fibrin clots infiltrated with canine neutrophils. These were generated by placing fibrin coated glass coverslips over denuded areas of skin created by Rebuck's skin window technique. An inflammatory infiltrate, consisting almost entirely of neutrophil and eosinophil granulocytes, was present in the fibrin matrix.[8] Indirect immunofluorescence microscopy revealed diffuse intracytoplasmic staining for fibrinogen in these neutrophils, whilst electron microscopy, demonstrated neutrophil internalisation of fibrin via extrusion of pseudopodia into the fibrin mass isolating smaller fibrin particles.[8] Lewis et al,[9] studied neutrophil mediated fibrinolysis in glycogen stimulated dog peritoneal neutrophils incubated with particulate fibrin and, using electron microscopy, demonstrated internalisation and degradation of fibrin particles.[9] The present study, based on that of Adams et al,[4,7] studied fibrinolysis by purified human neutrophils incorporated into polymerising fibrin clots. Only a very small percentage of neutrophils were demonstrated to internalise fibrin over a period in which complete dissolution of fibrin was achieved. Thus, whilst fibrin internalisation may contribute to neutrophil mediated fibrinolysis under certain experimental conditions,[8,9] this does not appear to occur under the conditions employed in the present study.

During the course of this study, PMA stimulated neutrophils were noted to achieve complete fibrin dissolution over a time period in which no appreciable changes were observed in the appearance of clots containing non-stimulated neutrophils. These observations were confirmed at an ultrastructural level by scanning electron microscopy which showed PMA stimulated neutrophils to cause complete destruction of the fibrin matrix at 60 minutes whilst non-stimulated neutrophils had little appreciable effect.

Studies measuring the rate of [^{125}I]-labelled fibrin solubilisation also revealed neutrophil mediated fibrinolysis to be upregulated following stimulation with PMA. At 90 minutes PMA stimulated neutrophils achieved full solubilisation of the fibrin clot whereas non-stimulated neutrophils achieved less than 50% solubilisation. The solubilisation of [^{125}I]-labelled fibrin by non-stimulated neutrophils was far higher than suggested by macroscopic observations and scanning electron microscopy. A closer consideration of the experimental design used to quantify [^{125}I]-labelled clot solubilisation may explain this discrepancy. The design is such that [^{125}I]-labelled fibrinogen (under the action of thrombin) is allowed to polymerise around neutrophils in the reaction mixture. It has been assumed that a measure of clot solubilisation can be made by subtracting the radioactivity not incorporated into a cell free (control) clot from the radioactivity released from a clot by incorporated neutrophils. However this may not represent an accurate measure of the neutrophil mediated clot solubilisation since neutrophils (non-stimulated and stimulated) cleave fibrinogen to a non-clottable form.[10] Fibrinogenolysis occurs very quickly and *minor* alterations in molecular weight profoundly affect fibrinogen clottability.* Thus in the presence of neutrophils, both clot formation and the generation of non-clottable fibrinogen are competing. The generation of non-clottable fibrinogen is impossible to quantify but the soluble radioactivity monitored during clot

* Degradation of fibrinogen (340 kDa) to a 330 kDa fragment is reported to be associated with 60% increase in the thrombin clotting time (a measure of the time required for fibrin formation), whilst further proteolysis to a 310 kDa fragment increases the thrombin clotting time by over 700%.[10] Analysis of earlier data, suggests that, under the conditions employed in the current study, neutrophil mediated proteolysis (by both non-stimulated and PMA stimulated neutrophils) may have a significant effect on fibrinogen clottability as early as 5-10 minutes following its exposure to neutrophils.[5,10]

dissolution by neutrophils is a composite of (1) non-incorporated fibrinogen, (2) non-clottable fibrinogen products and (3) solubilised fibrin. Thus, merely subtracting the soluble radioactivity of the *cell free* control clot may provide a falsely high measure of fibrin dissolution by incorporated neutrophils.

As PMA stimulated neutrophils have been shown to generate non-clottable fibrinogen degradation products more quickly than non-stimulated neutrophils,[9] the accuracy of quantification of [^{125}I]-labelled fibrinolysis might be worse affected in the case of PMA stimulated cells. It might therefore be argued that the lower density of clot around PMA stimulated neutrophils (observed macroscopically and by scanning EM) at 60 minutes may, in part, reflect an initial decreased availability of *clottable fibrinogen* rather than digestion of the fibrin clot. The initial density of the clot might also affect the speed of fibrin dissolution but the experiments conducted in this study could not assess this. Recent reports suggest that release of HNE in response to low dose PMA is time dependent.[11] Therefore the HNE content in the extracellular medium of PMA stimulated and non-stimulated neutrophils may not be that different during the period in which the rate of fibrin polymerisation is maximal (0-10 minutes - see figure 4b stippled line). Moreover, it is also known that ligation of integrin receptors on activated neutrophils by ECM proteins is a powerful stimulus for granule release.[12] Therefore the stimulatory effect of PMA on azurophil granule release might only be maximal once the fibrin matrix has formed. The extensive clot formation seen around neutrophils at 15 minutes (both macroscopically and by scanning EM) which was similar for PMA stimulated and non-stimulated neutrophils, would support this suggestion.

Whatever the mechanisms involved, it is clear that fibrin clots formed around PMA stimulated neutrophils are dissolved far more rapidly than those formed around non-stimulated neutrophils. These findings suggest the importance of neutrophil activation in fibrin(ogen)olysis under these conditions. It should be mentioned, however, that whilst non-stimulated neutrophils had little effect on fibrin clot size or structure during the period under investigation (90 minutes), these cells were eventually able to effect fibrin dissolution,

usually after 3-4 hours (as judged macroscopically). This fibrinolysis was most likely the consequence of neutrophil activation which is known to occur in isolated neutrophils during prolonged incubation at 37°C.[13] In addition, it is also possible that fibrinopeptide B (a known chemotactic factor for neutrophils)[14] released by thrombin, may contribute to neutrophil activation over time, as may thrombin itself.[15] Moreover, any neutrophil activation that does occur is likely to be amplified by ligation of integrin receptors following interaction with the fibrin matrix, a process known to trigger neutrophil activation responses.[12,16,17] Such activation may trigger the mobilisation and release of azurophil granules with an associated increase in fibrin degrading activity. Indeed, the periodic release of individual azurophil granules (with accompanying bursts of proteolytic activity) has recently been documented in neutrophils incubated at 37°C in fibronectin coated wells, in the absence of any exogenous stimuli.[18] It is possible that similar events may account for the fibrinolytic activity of non-stimulated neutrophils incorporated within fibrin matrices.

The relatively low capacity of non-stimulated neutrophils to effect fibrinolysis compared to that reported in previous studies[4,7] may reflect differences in the neutrophil activation state following isolation procedures. In the present study, a number of adaptations were made to neutrophil isolation procedures employed in previous studies in order to minimise the neutrophil activation that can occur during these procedures.[13,19] These included the use of autoclaved glassware and pipette tips as well as sterile filtered buffers. In addition, all steps following Ficoll centrifugation and dextran sedimentation, were performed at 4°C to minimise neutrophil activation.

It was possible that the mechanism by which neutrophil activation accelerated clot lysis was through the release of azurophil granule proteases. Concurrent studies had just revealed that stimulation with a low dose of PMA was capable of inducing release of azurophil granule proteases (chapters 4 and 5), and that these proteases were most likely responsible for the proteolytic activity of the neutrophil conditioned medium previously ascribed to a novel membrane associated protease.[4-6] Transmission electron microscopy was thus

undertaken to compare the granule content of fibrin entrapped neutrophils in the presence and absence of PMA. This revealed a marked decrease in the density of cytoplasmic granules in PMA stimulated compared to non-stimulated neutrophils. The present study did not attempt to distinguish between the various granule subsets, and therefore cannot conclude that the granules released necessarily included azurophil granules. These findings are, however, in conflict with earlier studies reporting no morphological evidence of degranulation under these conditions.[7] This is important, as these observations had been used to exclude the release of azurophil granule proteases under these conditions, allowing neutrophil mediated fibrinolysis to be solely ascribed to a membrane associated protease.[4,7]

This membrane associated proteolytic activity had been further distinguished from that of azurophil granule proteases on the basis of the fibrin degradation products that it generated, which had distinctly different apparent molecular mass values to those generated by preparations of granule proteases.[4,7] The present study showed, however, that when sufficiently diluted, preparations of granule proteases generated fibrin degradation products with identical apparent molecular mass values to those produced by a frequently used source of the membrane associated protease. It thus appears that the previously reported neutrophil mediated fibrinolysis,[4,7] is due to azurophil granule proteases released as a result of neutrophil activation.

Finally, it has been reported that egress of proteolytic activity from PMA stimulated neutrophils is enhanced in the presence of fibrin, and that this effect can be blocked by monoclonal antibodies directed against the integrin receptor CD11c/CD18.[7] The present study suggests that these findings most likely reflect the enhanced mobilisation and release of azurophil granules from PMA stimulated neutrophils following ligation of β_2-integrin receptors by the fibrin matrix.

Summary and conclusions

Neutrophil mediated fibrinolysis, under the conditions employed in this study, is predominantly an extracellular event occurring both at the neutrophil membrane and in the surrounding medium. It is markedly upregulated following activation with PMA, most likely on the basis of azurophil granule protease release. Fibrinolysis by non-stimulated neutrophils is far slower than with PMA stimulated cells, and is most likely due to partial activation of these cells during isolation procedures and during prolonged incubation at 37°C. Such activation, may be augmented via interactions with the fibrin matrix and can result in release of azurophil granules with subsequent degradation of the fibrin matrix. These findings are in keeping with biochemical evidence presented in chapter 5 suggesting that the *fibrinogen* degrading activity of the neutrophil membrane is due to azurophil granule proteases.

References

1. Adams SA, Kelly SL, Kirsch RE, Shephard EG. (1998) *Blood Coag. Fibrinol.* **9**, 307.
2. Plow EF, Edgington TS. (1978) The fibrinolytic pathway of leukocytes. From Neutral proteases of human polymorphonuclear leukocytes (Havemann K, Janoff A, eds), pg 330-45. Urban and Schwartzenberg, Baltimore.
3. Moroz LA. (1984) *Semin. Thromb. Haemostas.* **10**, 80.
4. Adams SA, Kelly SL, Kirsch RE, Robson SC, Shephard EG. (1995) *Blood Coag. Fibrinol.* **6**, 693.
5. Kelly SL, Adams SA, Robson SC, Kirsch RE, Shephard EG. (1994) *Biochem. J.* **298**, 689.
6. Shephard EG, Kelly SL, Anderson R, Fridkin M. (1992) *Clin. Exp. Immunol.* **87**, 509.
7. Adams SA. (1996) Proteases of the neutrophil membrane represent an alternative fibrinolytic pathway to that mediated by plasmin. (PhD Thesis, Univ. Cape Town).
8. Riddle JM, Barnhart JI. (1964) *Am. J. Pathol.* **45**, 805.
9. Lewis JH, Szeto ILF, Bayer WL, Curiel DC. (1972) *Blood.* **40**, 844.
10. Kelly SL. (1995) A neutral protease of the neutrophil surface: Role in the proteolyis of C-reactive protein and fibrinogen.(PhD Thesis, Univ. Cape Town)

11. Gaudin P, Berthier S, Barro C, Zaoui P, Morel F. (1997) *Eur. J. Cell. Biol.* **72**, 345.
12. Suchard SJ, Boxer LA (1994) *J. Immunol.* **152**, 290.
13. Berger M, O' Shea J, Cross AS, Folks TM, Chused TM, Brown EJ, Frank MM. (1984) *J. Clin. Invest.* **74**, 1566.
14. Senior RM, Skorgen WF, Griffin GL, Wilner GD. (1986) *J. Clin. Invest.* **77**, 1014.
15. Gillis S, Furie BC, Furie B. (1997) *Semin. Haematol.* **34**, 336.
16. Nathan C, Srimal S, Farber C, Sanchez T, Kabash L, Asch A, Gailit J, Wright SD. (1989) *J. Cell Biol.* **109**, 1341.
17. Berton G, Fumagalli L, Laudanna C, Sorio C. (1994) *J. Cell Biol.* **126**, 1111.
18. Liou TG, Campbell EJ. (1996) *J. Immunol.* **157**, 2624.
19. Fearon DT, Collins LA. (1983) *J. Immunol.* **130**, 370.

Chapter Eight

Summary and conclusions

This study aimed to purify a 600 kDa membrane associated protease reported to degrade fibrinogen, fibrin and C-reactive protein.[1-3] This protease, which had been implicated in a variety of biological processes,[1-8] appeared to be distinct from proteases of the azurophil granule on the basis of its molecular weight, its inhibitor profile and the nature of the products that it generated.[1-3]

The present study explored various avenues whereby this protease could be purified and further characterised. Although unsuccessful, several observations made during purification attempts led to the realisation that this proteolytic activity had similar properties to that of proteases of the azurophil granule.

- AEBSF affinity chromatography isolated proteolytic activity from soluble neutrophil cytoskeleton proteins that was identified by SDS-PAGE to contain proteins that had a mobility corresponding to that of proteases of azurophil granules.
- The conditioned medium from neutrophils stimulated with a low dose of PMA contained proteins with similar electrophoretic mobility to those of a conditioned medium known to contain azurophil granule proteases.
- The conditioned medium from neutrophils stimulated with a low dose of PMA generated products from plasminogen with a similar electrophoretic mobility to those generated by HNE.

When these results were considered together with reports on the existence of catalytically active, membrane-bound forms of HNE, cathepsin G, and proteinase 3,[9-13] the possibility that these proteases might be part of the proteolytic activity of the 600 kDa protease was considered. In this study poor solubility of lyophilised enzyme preparations was noted and this may account for the poor reproducibility of the migration of bands to regions of the gel previously reported to contain the proteolytically active subunits of the 600 kDa protease. This prompted the consideration that the

proteolytic activity in these preparations might represent aggregates of proteases of azurophil granule origin. It is known that the azurophil granule proteases are highly cationic and have a tendency to form heteroaggregates with negatively charged proteoglycans.[14]

Results from this study show quite clearly that the proteolytic activity previously ascribed to a 600 kDa protease is of azurophil granule origin. This conclusion is based on results of inhibitor profiles, the molecular size and hydrophobicity of the generated degradation products and peptides, and the molecular size of the proteolytic activity. The use of zymography as well as extensive experiments involving enzyme/substrate dose response studies and enzyme-inhibitor investigations were fundamental in obtaining the results that allowed this conclusion to be made. Previous studies analysed the molecular size of the protease by eluting the proteolytic activity from polyacrylamide gels after separation of concentrated enzyme preparations by SDS-PAGE.[1-3] In contrast, the present study employed zymography to determine the molecular mass of this proteolytic activity.[15] Due to the sensitivity of this technique, concentrating steps are not required prior to electrophoresis and therefore problems with aggregation of the enzyme species are overcome.

Fibrinogenolysis has been shown in this study to be predominantly due to HNE, with cathepsin G playing a lesser role and proteinase 3 also possibly involved. It is clear that the concentration of azurophil granule proteases determines the extent of fibrinogenolysis – i.e. the molecular size of the terminal degradation products is enzyme concentration dependent. In previous studies, where high concentrations of HNE were employed to degrade fibrinogen, it was concluded that since the electrophoretic mobility of the generated products was different from that of products generated by the proteolytic activity of the neutrophil membrane, membrane associated HNE was not responsible for fibrin(ogen)olysis.[2,3] Thus the size of the generated degradation products is not conclusive in deciding the nature of the protease involved until extensive enzyme-substrate dose response curves have been analysed. The low concentration of proteolytic activity in PMA stimulated neutrophil conditioned medium also accounts for the reported presence of HNE sensitive sites in what appeared to be the terminal fibrinogen degradation products generated by the conditioned medium.[2]

From this study it has also become apparent that the concentration of azurophil granule proteases determines the process of fibrinogenolysis. PMA stimulated neutrophil conditioned medium has been shown, in a previous study, to cleave fibrinogen from the N-terminus of the Aα chain and the C-terminal regions of Bβ and γ chains.[2] Other studies have reported the cleavage of fibrinogen from the N-terminus of all three chains by neutrophil lysates and pure HNE.[16-19] These apparent discrepancies in the process of fibrinogenolysis may well reflect the concentration of enzyme being used.

Concurrent with purification attempts, studies were undertaken to investigate one of the reported consequences of this membrane associated proteolytic activity. A previous study suggested that low molecular weight fibrinogen products (FDP) generated by the membrane protease could associate with neutrophil β_2-integrin receptors blocking subsequent adhesion to fibrinogen coated surfaces.[4] Thus it seemed that FDP might, through blockade of β_2-integrin receptors, modulate neutrophil responses at sites of inflammation. The present study aimed to confirm the association of FDP with integrin receptors.

Immunoelectron microscopy detected fibrinogen within the neutrophil and not on the membrane. This uptake process in the presence of high doses of fibrinogen (as used in previous studies investigating intact neutrophil processing of fibrinogen) is facilitated to a large extent by a non-specific pinocytic process. However an initial specific interaction with the neutrophil surface cannot be ruled out. The receptors involved in the putative interaction of fibrinogen with the neutrophil were not investigated in this study. However, fibrinogen has been shown in previous studies to effect the release of proteolytic activity (shown in this study to be azurophil granule proteases) from neutrophils which promotes its degradation in the extracellular medium.[4] It has been suggested that this interaction occurs near the CD11c/CD18 receptor as monoclonal antibodies directed against this receptor were found to cause marked inhibition of fibrin(ogen)olysis.[4] It is interesting to note that ligation of β_2-integrin receptors by soluble ICAM-1 has recently been reported to induce release of HNE from neutrophils.[20] This suggests that other soluble ligands for β_2-integrin receptors may also have the ability to induce azurophil granule release.

The inability to detect surface associated fibrinogen by immunoelectron microscopy might be due to a combination of its rapid internalisation and its rapid degradation by membrane associated azurophil granule proteases following its interaction with the surface. Such a notion is supported by previous reports that HNE proteolysis facilitates detachment of neutrophils bound to fibrinogen coated surfaces.[21]
It appears that neutrophils internalise both intact and degraded forms of fibrinogen. Degradation of these internalised proteins and partial release of low molecular weight fibrinogen degradation products into the extracellular medium then takes place. This route of fibrinogenolysis contributes minimally to the overall extent of the degradation process during incubation of fibrinogen with neutrophils as only a fraction of a percent of the fibrinogen in the surrounding medium is internalised by neutrophils under these conditions. The majority of neutrophil mediated fibrinogenolysis occurs thus via extracellular proteolysis, shown during this study to be due to azurophil granule proteases.

In view of the ability of PMA stimulated neutrophils to internalise and digest material in the pericellular environment, it was possible that similar endocytic activity might contribute to the process of fibrin clot lysis previously ascribed solely to the neutrophil membrane.[3] Having recently established techniques for the immunolocalisation of fibrinogen, these were applied to determine whether internalisation of fibrin played a significant role in neutrophil mediated fibrinolysis under these conditions. Immunoelectron microscopy revealed minimal evidence of fibrin internalisation during the period in which complete fibrinolysis occurred, supporting the earlier suggestion that neutrophil mediated fibrinolysis, under these conditions, is an extracellular event.

During this study it was noted that PMA stimulated neutrophils were able to achieve complete fibrin dissolution over a time period in which no appreciable changes were observed in the appearance of clots containing non-stimulated neutrophils. These macroscopic observations were supported both by studies measuring [^{125}I]-labelled fibrin solubilisation and by scanning electron microscopy. Scanning electron microscopy revealed morphological features of activation in

neutrophils stimulated with PMA, whilst transmission electron microscopy showed a decrease in the density of cytoplasmic granules in these cells with a redistribution of granules towards the periphery of the cytoplasm. This raised the possibility that clot lysis by PMA stimulated neutrophils might be mediated by the mobilisation of azurophil granules. Further studies revealed that, when used at sufficiently low concentrations, granule proteases generated fibrin degradation products with apparent molecular mass values identical to those generated by the conditioned medium of PMA stimulated neutrophils. Thus both morphological and biochemical evidence support a conclusion that the previously documented fibrin(ogen)olytic activity of the neutrophil membrane[2-5] is mediated by proteases of granule origin.

Previous studies have drawn attention to the important role played by the proteolytic activity of the neutrophil membrane which, through its ability to degrade fibrinogen, fibrin and C-reactive protein, may influence a number of biological processes.[1-8] The ability of membrane associated proteolytic activity to degrade fibrinogen and fibrin has emphasised the close relationship between the coagulation and inflammation pathways. The present study confirms the ability of the neutrophil to degrade fibrin(ogen), and provides biochemical and morphological evidence to suggest that this proteolytic activity is due to proteases of the azurophil granule. It has become increasingly apparent that membrane bound forms of azurophil granule proteases, through their ability to evade inhibition by naturally occurring protease inhibitors, may represent the biologically relevant forms of these proteases.[9-13] Therefore, taken together with studies which first identified the fibrinogen-, fibrin- and CRP-degrading activity of the neutrophil membrane, this study adds to the growing body of evidence that membrane bound forms of azurophil granule proteases may influence a variety of important biological processes.

References

1. Shephard EG, Kelly SL, Anderson R, Fridkin M. (1992) *Clin. Exp. Immunol.* **87**, 509.
2. Kelly SL, Adams SA, Robson SC, Kirsch RE, Shephard EG. (1994) *Biochem. J.* **298**, 689.
3. Adams SA, Kelly SL, Kirsch RE, Robson SC, Shephard EG. (1995) *Blood Coag. Fibrinol.* **6**, 693.
4. Kelly SL. (1995) A neutral protease of the neutrophil surface: Role in the proteolyis of C-reactive protein and fibrinogen.(PhD Thesis, University of Cape Town)
5. Adams SA. (1996) Proteases of the neutrophil membrane represent an alternative fibrinolytic pathway to that mediated by plasmin. (PhD Thesis, University of Cape Town).
6. Shephard EG, De Beer SM, Anderson R, Strachan AF, Nel AE, De Beer FC. (1989) *J. Immunol.* **143**, 2974.
7. Shephard EG, Anderson RA, , Rosen O, Myer MS, Fridkin M, Strachan AF, De Beer FC. (1990) J. Immunol. **145**, 1469.
8. Shephard EG, Anderson RA, Rosen O, Fridkin M. (1992) *Immunology.* **76**, 79.
9. Bangalore N, Travis J. (1994) *Biol. Chem. Hoppe Seyler.* **375**, 659.
10. Owen CA, Campbell MA, Sannes PL, Boukedes SS, Campbell EJ. (1995) *J. Cell Biol.* **131**, 775.
11. Owen CA, Campbell MA, Boukedes SS, Campbell EJ. (1997) *Amer. J. Physiol.* **272**, L385.
12. Gaudin P, Berthier S, Barro C, Zaoui P, Morel F. (1997) *Eur. J. Cell. Biol.* **72**, 345.
13. Csernok E, Ernst M, Schmitt W, Bainton DF, Gross WL. (1994) *Clin. Exp. Immunol.* **95**, 244.
14. Gullberg U, Andersson E, Garwicz D, Lindmark A, Ohlsson I. (1997) *Eur. J. Haematol.* **58**, 137.
15. Lantz MS, Ciborowski P. (1994) *Meth. Enzymol.* **235**(A) Chapter 45.
16. Plow EF, Edgington TS. (1975) *J. Clin. Invest.* **56**, 30.
17. Gramse M, Bingenheimer C, Schmidt W, Egbring R, Havemann K. (1978) *J. Clin. Invest.* **61**, 1027.
18. Bilezikian SB, Nossel HL. (1977) *Blood.* **50**, 21.
19. Gramse M, Bingenheimer C, Havemann K. (1980) *Thromb. Res.* **19**, 201.
20. Barnett CC, Moore EE, Mierau GW, Partrick DA, Biffl WL, Elzi DJ, Silliman CC. (1998) *Am. J. Physiol.* **274**, C1634.
21. Cai T-Q, Wright SD. (1996) *J. Exp. Med.* **184**, 1213.

Appendix 1: Chemicals & Reagents

All chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) except for those listed below.

Iodination

[^{125}I]-Na was purchased from Amersham (Buckinghamshire, UK), and iodogen from Pierce and Warinner (Cheshire, UK).

Neutrophil isolation

Dextran T-500 was obtained from Pharmacia (Uppsala, Sweden). Hanks balanced salt solution (HBSS) was obtained from Gibco/Life Technologies (Paisley, Scotland) and HEPES buffered HBSS was obtained from Highveld Biological (Johannesburg, South Africa).

Affinity chromatography

Tosylactivated superparamagetic polystyrene beads (Dynabeads®) were obtained from Dynal (Oslo, Norway). Affigel 10 affinity matrix was obtained from Biorad (California, USA). AEBSF ([2-aminoethyl]-benzenesulphonyl fluoride) was obtained from Boehringer Mannheim (Mannheim, Germany).

Molecular biology

Oligonucleotide probes for the screening of cDNA libraries were synthesised by Genosys Biotechnologies (Cambridgeshire, UK). A human bone marrow cDNA library, together with the E. Coli strain K802, were purchased from Clontech laboratories (California, USA). Hybond-N^{TM} nylon membranes, [γ-^{32}P]ATP and T4 polynucleotide kinase were obtained from Amersham (Buckinghamshire, UK). Sephadex PD-50 was obtained from Pharmacia (Uppsala, Sweden), Formamide was from Merck (Darmstadt, Germany).

Polyacrylamide gel electrophoresis and transblotting

A kit containing pre-stained molecular weight markers for use in SDS-PAGE was purchased from Amersham (Buckinghamshire, UK). This kit contained myosin (200 kDa), phosphorylase b (97 kDa), bovine serum albumin (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (21 kDa) and lysozyme (14

kDa). For autoradiography, Kodak XAR-5 film (Eastman Kodak, Rochester, NY) was used. Silver staining was performed using a silver staining kit obtained from Amersham (Buckinghamshire, UK). Polyvinylidene difluoride (PVDF) protein sequencing membrane (Immobilion™-P^{SQ}) was purchased from Millipore Corporation (Bedford, USA).

Fibrinogen proteolysis

The cathepsin G inhibitor, Z-Gly-Leu-Phe-CMK was purchased from Enzymes Systems Products (Livermore, USA). Chymostatin was obtained from Boehringer Mannheim (Mannheim, Germany). Human neutrophil elastase was purchased from Calbiochem (San Diego, USA). Acetonitrile was from Baxter Healthcare Corporation (Michigan, USA). Diethyl ether and LiChrosorb RP-18 were obtained from (Merck).

Immunoelectron microscopy and immunohistochemistry

Rabbit anti-human fibrinogen antibody (purified IgG fraction) and non-immune rabbit IgG were a kind gift from Brent Jennings (MRC/UCT Liver Research Centre, Cape Town, South Africa). Mouse monoclonal anti-CD11b and anti-CD18 were obtained from Sanbio (Uden, Netherlands). 30nm-gold conjugated antimouse IgG antibody and 10nm-gold conjugated streptavadin were obtained from Amersham (Buckinghamshire, UK), 15nm-gold conjugated goat antibiotin antibody was from Nanoprobes (New York, USA), 5nm-gold conjugated goat antirabbit IgG antibody was from Zymed (San Fransisco, USA). Streptavadin, biotinylated swine antirabbit IgG and horseraddish peroxidase conjugated streptavadin were obtained from Dako (Glostrup, Denmark). Bovine serum albumin was obtained from Seravac (Cape Town, South Africa). Hydrogen peroxide, sucrose, gelatin (microbiology grade), glutaraldehyde 25% (electron microscopy grade) and paraformaldehyde (electron microscopy grade) were obtained from Merck (Darmstadt, Germany). Spurr's resin was obtained from Agar Scientific Limited (Stanstead, UK).

Appendix 2: Abbreviations

AEBSF	(2-aminoethyl)-benzenesulphonyl fluoride
ATP	adenosine triphosphate
BSA	bovine serum albumin
bBSA	biotinylated bovine serum albumin
CRP	C-reactive protein
C3b	3rd component of complement, fragment b
C3bi	3rd component of complement fragment b (inactivated)
C5a	5th complement component, fragment a
CR1	complement receptor 1
CR3	complement receptor 3
EC	endothelial cell
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EM	electron microscopy
FBGN	fibrinogen
FDP	fibrin(ogen) degradation product
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FNNLP	N-formyl-norleucyl-leucyl-phenylalanine
HBSS	Hanks balanced salt solution
HEPES	N-[2-Hydroxyethyl]piperazine-N′-[2-ethanesulphonic acid]
H/HBSS	HEPES (1g/l) buffered HBSS
HNE	human neutrophil elastase
HOCl	hypochlorous acid
H_2O_2	hydrogen peroxide

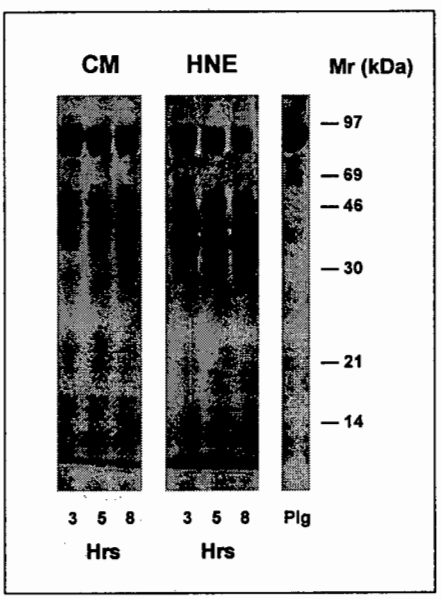
HPLC	high performance liquid chromatography
HSA	human serum albumin
IgG	immunoglobulin G
ICAM	intercellular adhesion molecule
IL-1	interleukin-1
IL-6	interleukin-6
IL-8	interleukin-8
kDa	kilodaltons
LAD	leukocyte adhesion deficiency
LFA-1	lymphocyte function associated antigen 1
LRI	leukocyte response integrin
LPS	lipopolysaccharide
LTB_4	leukotriene B_4
MMP	matrix metalloproteinase
MPO	myeloperoxidase
M_r	molecular weight
NADH	nicotinamide adenine dinucleotide phosphate
O_2^-	superoxide anion
PAF	platelet activating factor
PAI	plasminogen activator inhibitor
PBS	phosphate buffered saline
PBS/A	phosphate buffered saline containing BSA
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocyte
PMSF	phenylmethylsulphonyl fluoride
PR3	proteinase 3
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis

SBBG	streptavadin biotin bridge gold
TCA	trichloroacetic acid
TNF	tumour necrosis factor
t-PA	tissue plasminogen activator
u-PA	urokinase-type plasminogen activator

Appendix 3: Additional experimental data

Chapter 4 – Proteolysis of plasminogen

Proteolytic modification of plasminogen by pancreatic elastase has been reported to generate proteolytic fragments which have anti-angiogenic effects.[1,2] As part of the further characterisation of the proteolytic potential of the 600 kDa neutrophil membrane protease, studies were undertaken to investigate whether it was capable of similar proteolytic modification. Preliminary experiments revealed the neutrophil conditioned medium to generate several products within the 45 kDa region of the gel. N-terminal sequencing of these products identified a fragment containing the kringle 5 domain of plasminogen, which is known to have anti-angiogenic activity.[2] In one experiment, the pattern of plasminogen degradation by the conditioned medium was compared to that of purified HNE. Human plasminogen (62.5μg) was incubated (37°C, 3-8 hours) with either the conditioned medium from 10^6 PMA (5ng/ml) stimulated neutrophils, in a final volume of 200μl (*left hand panel in gel opposite*) or with purified HNE (0.3μg) in a final volume of 100μl (*right hand panel*). As a control, plasminogen was incubated at 37°C for 8 hours in the absence of proteases (*far right hand lane*). It can be seen that the products generated by these two sources had similar apparent molecular mass values.



References

1. Cao Y, Ji R-W, Davidson D, Schaller J, Marti D, Sohndel S, McCance Cao Y, O'Reilly MS, Llinas M, Folkman J. (1996) *J. Biol. Chem.* **271**, 29461.
2. Cao Y, Chen A, Soo S, Ji R-W, Davidson D, Cao Y, Llinas M. (1997) *J. Biol. Chem.* **272**, 22924.

Chapter 5 – Additional experimental data

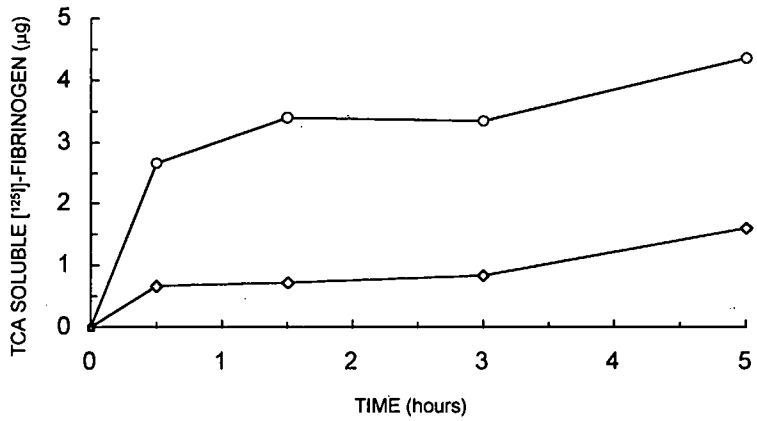


Figure 1 (see page 132): The effect of MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl on the [^{125}I]-labelled-fibrinogen degrading activity of the conditioned medium. *(a) [^{125}I]-labelled fibrinogen (10µg) was incubated (37°C, 1-5 hours) with the conditioned medium from 10^6 PMA (5ng/ml) stimulated neutrophils in a final volume of 100µl. Reactions were performed in the absence (-O-) and presence (-◊-) of MeO-Suc-(Ala)$_2$-Pro-Val-CH$_2$Cl (1mM). Degradation was measured as 10% (w/v) TCA soluble peptide formation. Each data point represents a single measurement. This data is also presented in figure 6b page 139.*

Chapter 6 – Additional experimental data

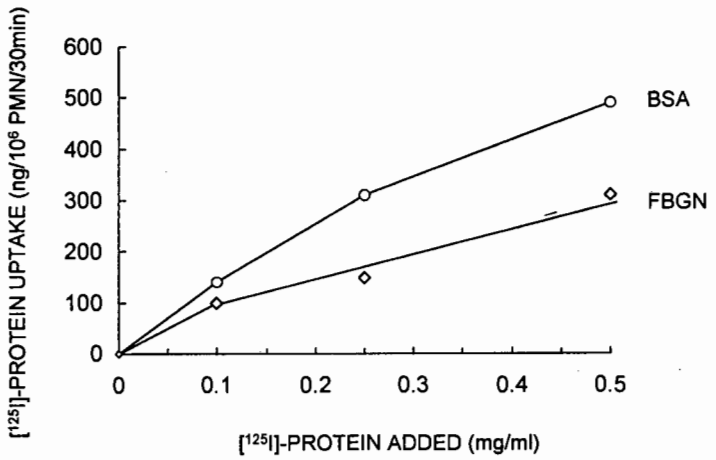


Figure 5a (see page174) : Association of [^{125}I]-labelled fibrinogen and BSA with PMA stimulated neutrophils (PMN), as a function of concentration. *[^{125}I]-labelled fibrinogen (FBGN) or [^{125}I]-labelled BSA (100-500μg/ml) were incubated (37°C, 30 minutes) with PMA stimulated PMN (5 x 10^6/ml) in PBS. Association of [^{125}I]-labelled protein was measured and expressed as ng [^{125}I]-labelled protein per 10^6 PMN. Each data point represents a mean of duplicates.*

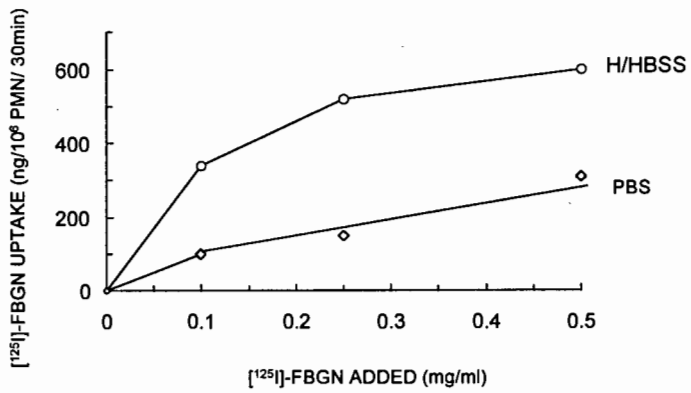


Figure 9a (see page 178): The effect of divalent cations on [^{125}I]-labelled fibrinogen association with PMN. *(a) [^{125}I]-labelled fibrinogen (100-500μg/ml) was incubated (37°C, 30 minutes) with PMA stimulated PMN (5 x 10^6/ml) in either PBS or HEPES buffered HBSS (H/HBSS). Association of [^{125}I]-labelled fibrinogen was measured and expressed as ng per 10^6 PMN. Each data point represents a mean of duplicates.*

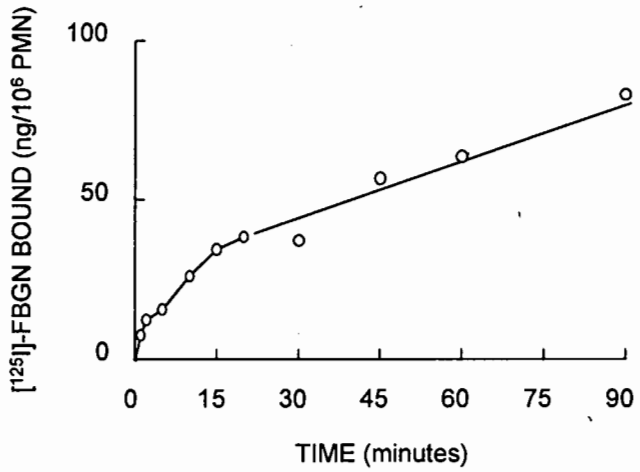


Figure 11 (see page 180): Association of [^{125}I]-labelled fibrinogen with chilled PMN as a function of time. *Chilled PMA stimulated PMN (prepared as described in figure 10) were incubated with [^{125}I]-labelled fibrinogen (100μg/ml) on ice over a time course of 0-90 minutes. Association of [^{125}I]-labelled fibrinogen was measured and expressed as ng per 10^6 PMN. Each data point represents the mean of duplicates.*

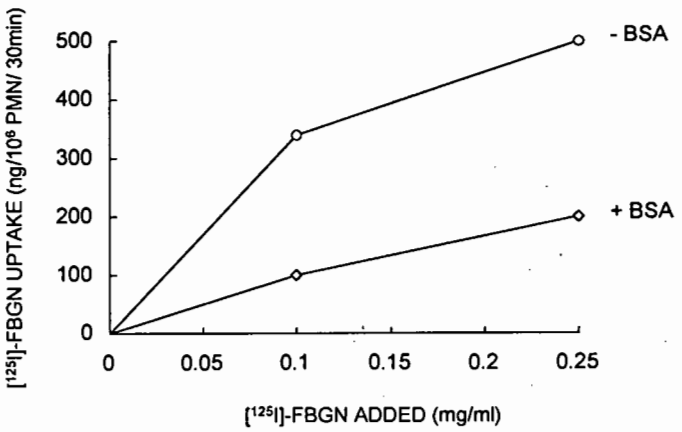
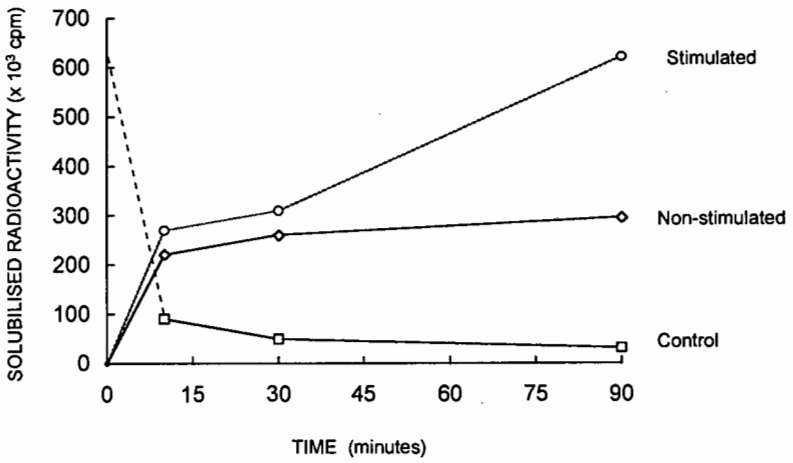


Figure 13 (see page 182): The effect of BSA on the association of [^{125}I]-labelled fibrinogen with PMN. *[^{125}I]-labelled fibrinogen (100 and 250μg/ml) was incubated (37°C, 30 minutes) with PMA stimulated PMN (5 x 10^6/ml) in the presence and absence of BSA (2mg/ml). Association of [^{125}I]-labelled fibrinogen was measured and expressed as ng per 10^6 PMN. Each data point represents the mean of duplicates.*

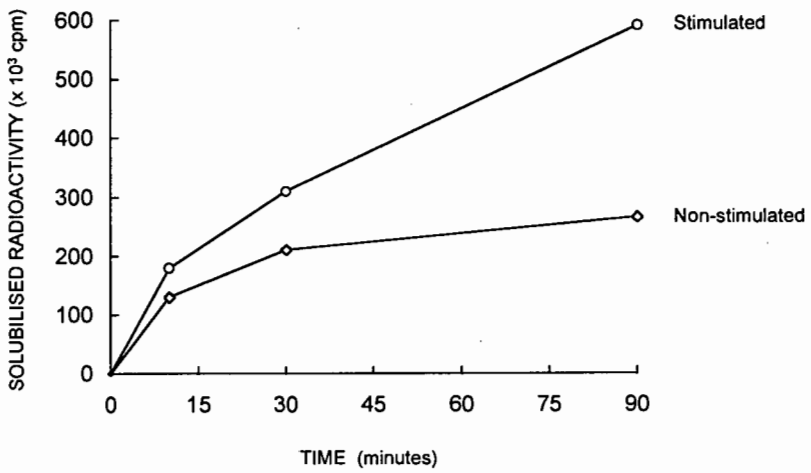
Chapter 7 – Additional experimental data

Experiment 1

(a)

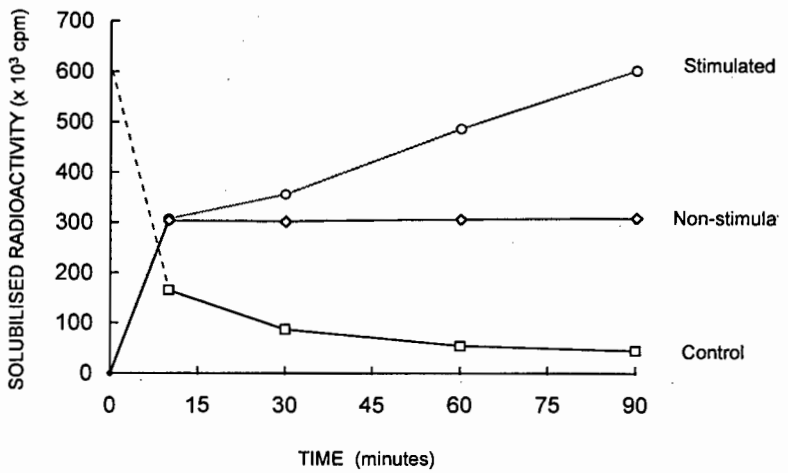


(b)



Experiment 2

(a)



(b)

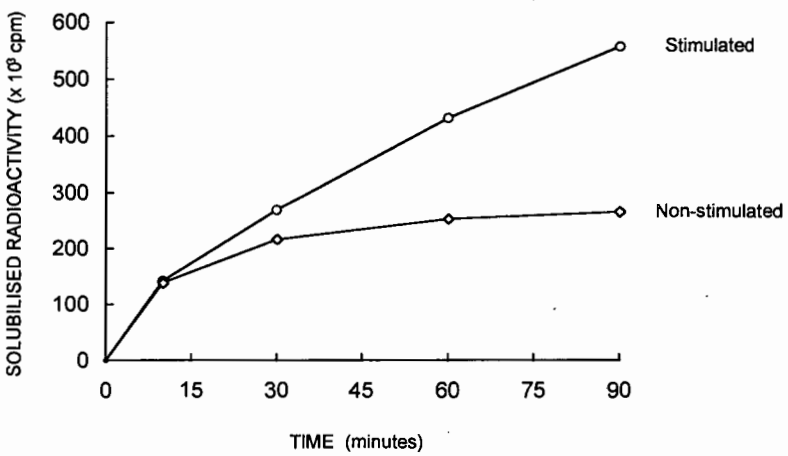


***Figure 4 (see page 205)**: Comparison of [^{125}I]-labelled fibrin solubilisation by non-stimulated and PMA stimulated neutrophils. (Additional experiments 1 and 2) (a) Non-stimulated or PMA (8ng/ml) stimulated neutrophils (10 x 10^6/ ml) were incorporated (37°C, 10-90 minutes) into 1mg/ml [^{125}I]-labelled fibrin clots. At selected time points solubilised radioactivity was measured and expressed as cpm released. The soluble radioactivity associated with cell free control clots was also measured at each time point. The progressive decrease in the soluble radioactivity seen in cell free control clots reflects fibrinogen polymerisation. (b) This figure shows the radioactivity released by stimulated and non-stimulated neutrophils experimental samples following the subtraction of radioactivity associated with the cell free control at each time point. Data points represent means of duplicates.*