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PROTEASES OF THE NEUTROPHIL MEMBRANE REPRESENT AN ALTERNATIVE FIBRINOLYTIC PATHWAY TO THAT MEDIATED BY PLASMIN

by

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for my parents

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If you have built castles in the air, your work need not be lost; that is where they should be. Now put the foundations under them.

Henry David Thoreau

Abstract

Proteases of the neutrophil membrane represent an alternative fibrinolytic pathway to that mediated by plasmin

The cellular components of the blood, which become associated with fibrin through specific cellular adhesive processes, play a significant role in the breakdown of fibrin. Fibrinolysis by elastase and cathepsin G, enzymes present within the azurophilic granules of the neutrophil, has previously been shown. Recent studies have demonstrated neutrophil-mediated fibrinogenolysis by a membrane-associated protease which suggests that proteases connected with the neutrophil membrane might also be capable of clot dissolution. Investigations showed that neutrophil-mediated clot lysis was effected by a membrane-associated serine protease that can be dissociated by SDS-PAGE to bands that migrate to apparent molecular weights of 501 kDa, 398 kDa, 316 kDa, 245 kDa and 209 kDa. This degradation was distinct from that produced by plasmin, neutrophil lysosomal enzymes and purified human neutrophil elastase and enhanced the action of plasmin in clot solubilization. Preincubation of neutrophils with monoclonal antibodies directed against the CD11c/CD18 integrin was able to significantly inhibit neutrophil membrane-dependent fibrinolytic activity. Upregulation of enzyme activity occurred following association of fibrin substrate with the cell membrane and was dependent on the activation of cellular kinases, in particular protein kinase C.

Fibrin products generated by neutrophil membrane proteolytic activity were found to possess biological activity. The low molecular weight peptides effected substantial inhibition of thrombin-induced platelet aggregation while the presence of the higher molecular weight material could partially overcome platelet-induced resistance to plasmic lysis. No modulation of platelet-mediated fibrin clot retraction was observed using these same fibrin products.

Neutrophil lysosomal enzyme activity was shown to further degrade the end products of plasmic fibrin degradation into low molecular weight material, followed by reassembly of higher molecular weight products in a process dependent on calcium and factor XIII. The reformed products have a similar molecular weight to those produced by plasmic lysis of fibrin, as well as a putative crosslinked site. However, the isoelectric point of these reformed products indicates they are distinctly different from plasmin-derived fibrin products. These reassembled products were recognized by a monoclonal antibody raised against D-dimer. Processing by neutrophils of the end products of plasmic fibrin degradation may have the potential for modulating the immune response as well as compromising the predictive value of tests measuring D-dimer, used as a laboratory marker of a number of thromboembolic disorders encountered in clinical practice.

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CHAPTER 1

Introduction

Blood coagulation and the fibrinolytic system comprises a vast array of finely controlled biochemical pathways. Clot formation and clot lysis are essential initial and final components respectively of haemostasis, the cessation of bleeding that follows traumatic interruption of vascular integrity. Vascular damage alters the normal anticoagulant surface of the endothelial cells to a procoagulant surface. Concomitantly endothelial cells acquire adhesive properties for leukocytes and platelets, due to upregulated expression of endothelial cell adhesion molecules. These processes are a consequence of endothelial cell surface expression of tissue factor during vascular injury. Tissue factor is known to play a major role in promoting coagulation, through activation of coagulation factors which leads ultimately to the generation of thrombin, a serine protease and a key enzyme in the haemostatic process. Cleavage of the soluble plasma glycoprotein fibrinogen by thrombin is the initial step in the formation of insoluble fibrin polymer clot. In addition thrombin activates platelets and is involved in the upregulation of platelet and endothelial cell P-selectin which supports leukocyte adhesion to these cells. Thus fibrin deposition and accumulation of platelets and leukocytes at sites of vascular injury is a dynamic process.

With regard to fibrinolysis the serine protease plasmin, arising through the action of plasminogen activator cleavage of the proenzyme plasminogen, is considered to be the major enzyme participating in fibrinolysis. Plasmin catalyses the degradation of fibrin to a number of predictable and well described end products (Pizzo *et al*, 1973). The knowledge that granulocytes are not merely trapped within the developing thrombus but are present due to specific cellular adhesive processes (Palabrica *et al*, 1992) suggests that there may be important physiological repercussions to their presence within thrombi. It has been recognized since the turn of the century that leukocytes, in particular neutrophils, play a role in fibrinolysis (Rulot, 1904; Opie, 1907). The entire fibrinolytic potential of the neutrophil has been ascribed to the proteases cathepsin G and elastase, found within the intracytoplasmic azurophilic granules of the neutrophil and requiring high activation states of the cell to be released (Moroz, 1984; Plow and Edgington, 1978). Little consideration has been given to the importance of membrane-associated proteases, which are in direct contact with fibrin substrate and thus would be expected to be far more readily mobilised than elastase and cathepsin G.

The experimental section of this thesis is aimed at investigating the contribution of proteases associated with the neutrophil membrane to the fibrinolytic pathway. This process will be compared to the classical plasmin-mediated degradation pathway as well as that of the lysosomsal enzymes, previously assesssed as being soley responsible for the ability of the neutrophil to digest fibrin. These studies were prompted by the recently published data showing that a 600 kDa membrane-associated neutrophil protease is capable of digesting fibrinogen in a manner distinct from that of plasmin and neutrophil lysosomal enzymes (Kelly *et al*, 1994). This protease is also capable of processing the acute phase reactant C-reactive protein to a number of bioactive peptides (Shephard *et al*, 1989).

The close association of neutrophils with fibrinogen has long been established and the role of the leukocyte specific β_2 integrins is of particular relevance (Wright *et al*, 1988; Altieri *et al*, 1990; Altieri *et al*, 1993; Diamond and Springer, 1993b). These adhesion molecules, together with the leukocyte response integrin, have been shown to specifically bind fibrinogen. In this manner "protected pockets" are formed, which allow released proteinases to exert their effects under relative protection from circulating proteinase inhibitors (Weitz *et al*, 1987). It has also become clear that the integrins act not only as adhesion molecules but are also essential in signal transduction pathways, both into and out of the cell. Ligand occupation of receptor triggers a series of intracellular events resulting in cytoskeletal changes, activation of various kinases, protein phosphorylation and resulting functional effects (Pavalko and La Roche, 1993; Pavalko and Otey, 1994; Wang *et al*, 1993; Petty and Todd, 1996). In light of this the mechanisms involved in membrane-associated fibrinolysis were investigated, with particular emphasis on the role of cellular kinases and the β_2 integrins.

In addition to the role of the fibrinogen molecule within the haemostatic and coagulation system, degradation of the parent molecule into fragments releases products with vasoactive properties and immunomodulatory functions. For the most part, the biological role of plasmin-derived fibrin degradation products have been investigated. This thesis aims to assess the importance of neutrophil membrane protease-derived fibrin degradation products in modulating platelet function. Following disruption of the vascular endothelial cell lining platelets adhere to the exposed subendothelial matrix and undergo a series of calcium dependent biochemical and structural changes. The functional activity of the platelet essentially involves four mechanisms: adhesion, aggregation, contraction and secretion. Fundamental to these changes is the binding of fibrin(ogen) to the platelet by the β_3 integrin receptor, GPIIb-IIIa. This occurs via specific regions

both in the fibrinogen molecule and the subunits of the GPIIb-IIIa receptor (Smith *et al*, 1990; Kloczewiak *et al*, 1982; Takada *et al*, 1992; Bajt *et al*, 1992; D'Souza *et al*, 1990). Peptides incorporating these specific recognition sequences have been found to act as both agonists and competitive antagonists of integrin function (Plow *et al*, 1987; Du *et al*, 1991). It is tenable therefore, that products released following neutrophil membrane proteolytic degradation of fibrin may have the ability to modulate platelet function. Studies on the resulting platelet aggregation and clot retraction in the presence of these products were therefore performed. Platelet rich clots have been found to be relatively resistant to plasmic lysis (Falk, 1992), probably on the basis of platelet production of plasminogen activator inhibitor-1 (Levi *et al*, 1992), increased clot retraction (Kunitada *et al*, 1992) and greater extent of crosslinking of the fibrin molecule (Reed *et al*, 1991). The role of the neutrophil in overcoming this inhibition together with the possible modulation by neutrophil membrane-derived fibrin degradation products in the fibrinolytic process was investigated. The theoretical basis behind this study was provided by Braaten *et al* (1994) who showed that the potent anti-adhesive peptide D-RGDW was able to "uncouple" fibrin from the platelet fibrinogen receptor and significantly increase the rate of plasmin-mediated fibrinolysis occuring at the platelet-fibrin interface. The use of neutrophil-derived products as opposed to synthetic peptides would provide a more realistic representation of what may occur in the *in vivo* situation.

Plasmin digestion of fibrin gives rise to a number of predictable end products which have biological activity. These range from inhibition of neutrophil oxidative metabolism (Kazura *et al*, 1989) and modulation of neutrophil endothelial cell interactions (Fischer *et al*, 1991) to effects on cell proliferation (Robson *et al*, 1993; Hatzfield *et al*, 1982) and the release of cell-derived growth-factors (Lorenzet *et al*, 1992). Significantly high concentrations of these products are found in the peripheral circulation of patients receiving exogenous thrombolytic drugs as treatment for pathological thromboembolic conditions such as myocardial infarction, pulmonary embolism and deep vein thrombosis. These therapeutic agents have also been found to significantly increase the number of circulating activated neutrophils (Ranjadayalan *et al*, 1991; Adams *et al*, 1995). Only two very recent publications have addressed the question of neutrophils, and more specifically human neutrophil elastase, secondarily digesting these plasmin-derived fibrin products (Leavell *et al*, 1996; Bach-Gansmo *et al*, 1996). This study attempts to investigate more closely the role of neutrophil lysosomal enzymes in degrading these products and to characterize the enzymatic and biochemical processes involved.

It is hoped that the studies undertaken will provide a greater understanding of neutrophil membrane fibrinolytic activity and its importance in controlling fibrin deposition and thrombus dissolution.

CHAPTER 2

Fibrinogen and fibrin

CHAPTER 2

Fibrinogen and fibrin

Introduction

Maintenance of the integrity of the vascular system plays a key role in survival. Central to this role is the plasma glycoprotein, fibrinogen, which participates in the final phase of the blood coagulation cascade. Elucidation of the function and structure of this remarkable molecule, which on exposure to the protease thrombin is able to transform from soluble fibrinogen within the plasma to a gel-like fibrin clot, the primary ingredient of blood clot, has occupied the energies and attention of scientists for many decades. Investigations have encompassed both the ambit of conventional protein chemistry and the rapidly emerging field of molecular biology. The proteolytic alteration of fibrinogen from a soluble molecule into fibrin is characterized by the formation of fibrin monomer, which can then link up with other monomers and form a long thread-like insoluble polymer, fibrin. Polymerized fibrin serves as a template for the localized assembly and activation of the fibrinolytic system which modulates fibrin deposition and clot dissolution. Binding of fibrinogen to vascular cells such as platelets supports platelet aggregation and to endothelial cells facilitates tissue repair. The interaction of fibrinogen with platelets and neutrophils will be discussed within the relevant sections in the literature review.

Approximately 75% of the body's fibrinogen is found within the plasma (Takeda, 1966) but it is also found distributed within the interstitial fluid and the lymph. Plasma fibrinogen is synthesized exclusively by hepatocytes at a rate of 1.7 to 5 g/day (Straub, 1963; Takeda, 1966) in the steady state situation, and has a half-life of 3 to 5 days once in the plasma (Collen *et al*, 1972). However, the synthetic reserve for fibrinogen is large and up to 20-fold increases in production may occur (Reeve and Franks, 1974) in response to a variety of stresses including inflammation, trauma and pregnancy (Aronsen *et al*, 1972). Fibrin degradation products (FDP's), in particular fragment D, appear to play a major role in controlling synthesis of fibrinogen by feedback mechanisms (Nham and Fuller, 1986). In a rat hepatocyte model this fragment interacts with peripheral blood monocytes/macrophages which in turn produce hepatocyte-stimulating factor (Ritchie *et al*, 1982) or interleukin-6 (IL-6) (Gauldie *et al*, 1987) which are able to increase fibrinogen synthesis. IL-6

is thought to activate an intracellular mechanism involving protein kinase C (PKC) to stimulate the production of the mRNAs for fibrinogen (Evans *et al*, 1987).

Platelets represent an additional fibrinogen source (Castaldi and Caen, 1965) and make up approximately 3% of the circulating plasma pool. Controversy exists regarding whether this fibrinogen is functionally, metabolically or structurally different from plasma fibrinogen (Doolittle *et al*, 1974; James *et al*, 1977). The site of platelet fibrinogen synthesis also remains a matter for debate. There is evidence to suggest that megakaryocytes may synthesize fibrinogen (Levin *et al*, 1985) but an increasing number of studies indicate that endocytosis of exogenous fibrinogen occurs with incorporation of exogenous fibrinogen into the platelet α-granules (Harrison *et al*, 1989). It appears that endocytosis may occur following binding of fibrinogen to the GPIIb-IIIa integrin receptor (see Chapter 4) (Harrison *et al*, 1990). This study was further substantiated by Handagama *et al* (1993) in experiments where kistrin was used to block endocytosis of fibrinogen into guinea pig megakaryocyte and platelet α-granules. Kistrin belongs to a group of integrin inhibitory proteins isolated from viper venoms termed disintegrins which contain the Arg-Gly-Asp sequence through which they bind to GPIIb-IIIa. The question of how fibrinogen is able to bind to unstimulated platelets and become endocytosed is controversial. It has been suggested that shear stress may induce fibrinogen binding to GPIIb-IIIa in the peripheral circulation, fibrinogen may become immobilised to biological surfaces and thereafter bind to GPIIb-IIIa or alternatively platelets may undergo cycles of activation within the circulation allowing them to bind fibrinogen (Handagama *et al*, 1995).

Fibrinogen is a large dimeric glycoprotein with a molecular weight of 340 kDa, which corresponds to a total of about 3000 amino acids. It is made up of three pairs of disulfide bonded polypeptide chains termed Aα (610 amino acids), Bβ (461 amino acids) and γ (441 amino acids) with molecular weights of 66 kDa, 52 kDa and 46.5 kDa respectively (McKee *et al*, 1966; Henschen *et al*, 1983; Doolittle, 1983). The six polypeptides are arranged into three globular domains, the central E-domain and two excentric and larger D-domains which are linked by a coiled-coil triple helix structure of 111 amino acids (Doolittle *et al*, 1978). The presence of the coiled-coil region of fibrinogen has important consequences for the assembly of the three chains, the mechanical properties of the resultant fibrin fibres and plasmic digestion of the fibrin clot (Hantgan *et al*, 1994). The computed molecular weight of the total molecule is therefore approximately 330 kDa. However, although the Aα chain contains no carbohydrate, the Bβ and γ chains have four carbohydrate sidechains linked through N-acetylglucosamine to asparagine 52 of

each γ chain and asparagine 364 of each Bβ chain (Mills and Triantaphyllopoulos, 1969; Blomback *et al*, 1973; Topfer-Peterson *et al*, 1976) which increases the molecular weight to 340 kDa (Scheraga and Laskowski, 1976).

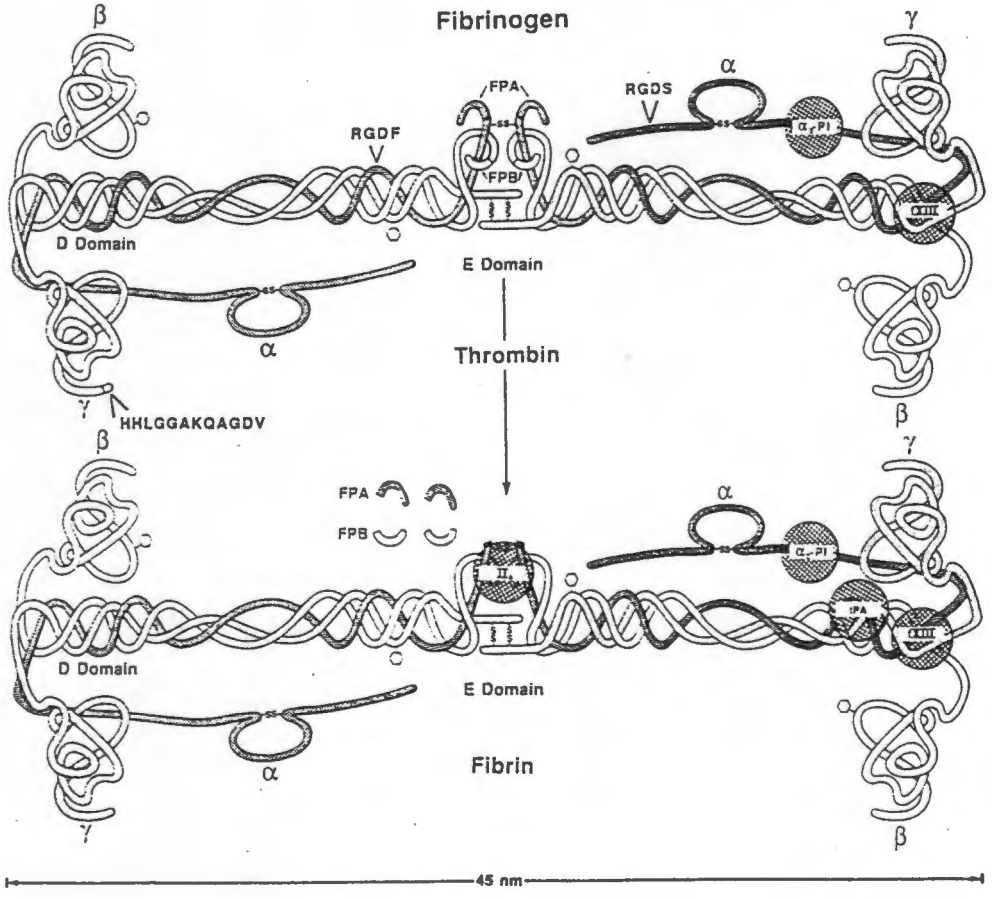
The three pairs of polypeptide chains of the fibrinogen molecule are held together by 29 disulfide bonds. This dimeric molecule consists of two half-molecules, with each half molecule containing the three nonidentical polypeptide chains. The two half molecules are joined by three symmetrical disulfide bonds between adjacent $A\alpha cys_{28}$ and γcys_8 and cys_9 (Blomback *et al*, 1976; Hoeprich and Doolittle, 1983) as well as a disulfide link between $A\alpha cys_{36}$ of one half molecule and $B\beta cys_{65}$ of the other half molecule (Huang *et al*, 1993). The central E domain contains the amino termini of the 6 polypeptide chains. The two terminal D domains are formed by globular carboxy terminal (C-terminal) domains of the Bβ and γ chains. The C-terminal globular domains of the larger Aα chains are thought to fold back and contribute to the structure of the central node (Weisel *et al*, 1986). The coiled-coil alpha-helical region of 111 amino acids is flanked, in each of the polypeptide chains, by 2 pairs of cysteines which are involved in interchain linkages within each half molecule and are termed disulfide rings (Doolittle *et al*, 1978). The disulfide rings which flank the coiled-coil region play an important role in the interchain assembly of the half molecules of fibrinogen as well as in the final dimer formation (Zhang and Redman, 1994). In addition there are several intrachain disulfide bonds. In all, 29 disulfide bonds (Blomback *et al*, 1968); 8, 11 and 10 cysteine residues on the Aα, Bβ and γ chains respectively with all of the 58 cysteine residues in the molecule participating in these 29 disulfide bonds (Henshen, 1964; Garlund *et al*, 1977). In all vertebrate species studied this complex disulfide-linked fibrinogen structure is essentially the same with a probable common assembly pathway (Oddoux and Grieninger, 1994).

The α, β and γ chains of the molecule are encoded by three independent genes present on chromosome 4 (Chung *et al*, 1983a, 1983b, 1990; Rixon *et al*, 1983; Kant *et al*, 1985). Extensive homology exists between the amino acid sequences of the three chains as well as between various species studied indicating that the present fibrinogen genes evolved from a common ancestral gene (Doolittle *et al*, 1979). The individual polypeptide chains are processed, glycosylated and assembled. This assembly into the fibrinogen complex occurs within the endoplasmic reticulum of the cell (Hurtley and Helenius, 1989) with Bβ chain synthesis appearing to be the rate limiting step in fibrinogen assembly (Roy *et al*, 1991). Newly synthesized Bβ chains are used and secreted more rapidly than Aα or γ chains and a large intracellular pool of free α and γ chains exists. The Bβ chains appear to bind to BiP, the immunoglobulin heavy chain binding protein, thought to act

as a so-called "molecular chaperone" within the endoplasmic reticulum. It is involved in the assembly of immunoglobulin chains as well as aiding in folding, assembly and oligomerization of proteins together with prevention of transport of malformed or aberrantly glycosylated proteins (Kassenbrock *et al*, 1988). The Bβ domain between amino acids 73 and 93 is necessary for the assembly of the three fibrinogen chains and marks the start of the alpha-helical "coiled-coil" region of fibrinogen (Zhang and Redman, 1992). In addition to disulfide bonds mentioned previously, noncovalent interactions of other amino-terminal amino acid residues in the three fibrinogen chains are also of importance in dimer formation (Zhang and Redman, 1996). Although surplus fibrinogen chains occur intracellularly within hepaocytes, only fully assembled fibrinogen is secreted (Plant and Grieninger, 1986). Degradation of surplus polypeptide chains of fibrinogen may occur in the endoplasmic reticulum or lysosomes of the cell (Kassenbrock *et al*, 1988).

Three high affinity binding sites for calcium have been characterized on human fibrinogen, two of which are associated with the D-domain while the site of the third remains controversial. Marguerie (1977) favoured a site formed by the two α-chain extensions while Niewenhuizen (1981) assigned the third site to the central domain. These calcium binding regions are fully occupied in circulating fibrinogen (Nieuwenhuizen *et al*, 1981). Low affinity calcium binding sites are also thought to exist, with evidence to suggest that sialic residues in human fibrinogen fulfil this role (Dang *et al*, 1989). These divalent ions play a major role in maintaining the structure and stability of fibrinogen. Millimolar calcium concentrations limit the extent of plasmin digestion of both fibrinogen and fibrin (Haverkate and Timan, 1977) and protect fibrinogen against denaturation by heat (Ly and Godal, 1973) and acidic pH (Margeurie, 1977). The presence of calcium is also able to accelerate fibrin formation (Boyer *et al*, 1972) and the mechanical strength of these fibrin clots, formed in the presence of millimolar concentrations of calcium, are considerably greater (Shen *et al*, 1975). In addition, the transglutaminase factor XIIIa, essential for covalent stabilization of the fibrin molecule is functionally dependent on the presence of calcium (Laudano and Doolittle, 1981).

Figure 1 Schematic model of fibrinogen and its conversion to fibrin monomer by the action of thrombin. The fibrinogen molecule consists of three pairs of polypeptide chains Aα, Bβ and γ. The central E domain contains the N-terminal regions of all six chains joined by disulfide bonds to form a dimeric structure. Conversion of fibrinogen to fibrin is initiated by the action of thrombin, leading to the release of fibrinopeptides A and B from the Aα and Bβ chain respectively. Also indicated are the binding sites on the molecule for IIa, t-PA, factor XIII and α_2-antiplasmin. The platelet binding sequences KQAGDV and RGD are shown. From Mosesson (1993).



Fibrin formation

The conversion of soluble fibrinogen into the insoluble fibrin polymer involves three steps: the thrombin catalyzed removal of fibrinopeptides, a non-covalent assembly process and covalent stabilization of fibrin by factor XIIIa-catalyzed crosslinking. In addition to the importance of fibrin in creating a haemostatic plug in trauma and acting as a temporary matrix in wound healing and inflammation, more recently other functions have been ascribed to this insoluble protein. Qi and Kreatzer (1995) were able to demonstrate that the presence of fibrin matrix in cultures of endothelial cells isolated from calf pulmonary endothelium was able to induce endothelial morphological changes. These included cell retraction and disorganization and occurred in a protein concentration dependent manner and were reversible on removal of the clot. In this same study fibrin was also shown to induce leukocyte chemotactic activity, possibly related to interleukin-8 (IL-8) expression in these vascular endothelial cells. Following thrombin-induced cleavage of fibrinopeptide B from fibrinogen, a fibrin-specific region is exposed which appears to play an important role in stimulating vascular cell responses. These include spreading of platelets and endothelial cells as well as the release of von Willebrand factor (vWf) (Ribes *et al*, 1987; Bunce *et al*, 1992; Hamaguchi *et al*, 1993). Sporn *et al* (1995) were able to show that cell proliferation, an important determinant of revascularization, requires exposure of this same fibrin-specific site. The biological role of FDP's will be discussed later on in this chapter.

The serine protease thrombin not only plays a central role in haemostasis but is also an activator of many cell-mediated events. Functional domains throughout the molecule are involved in the processes of cell adhesion, chemotaxis and proliferation (Bar-Shavit and Wilner, 1986; Bar-Shavit *et al*, 1992). It has been suggested that plasminogen activators associated with extracellular matrix may play an important role in converting the circulating zymogen prothrombin to thrombin. The thrombin generated also binds to extracellular matrix to exert functional effects involving cellular invasion, proliferation and growth (Benezra *et al*, 1993). Clot-bound thrombin is able to act as a reservoir for enzymatically active thrombin which is resistant to inactivation by circulating thrombin inhibitors (Weitz *et al*, 1990). This bound thrombin is able to activate factor XIII, necessary for covalent stabilization of the fibrin molecule (see next section).

Thrombin acts on fibrinogen to hydrolyse specific Arg-Gly bonds on the Aα and Bβ chains to release two peptides, fibrinopeptide A and B (Blomback, 1958). Thrombin specificity appears to be due in part to the presence of a set of hydrophobic residues in the Aα chain ($A\alpha_{16\text{-}17}$) of fibrinogen which bind with a complementary apolar region on thrombin. This interaction positions

the Arg_{16}-Gly_{17} peptide bond of the Aα chain within the catalytic site of thrombin (Marsh *et al*, 1983; Ni *et al*, 1989). The active site within the thrombin molecule can be considered as two separate domains: a catalytic site that includes the Arg-His-Ser catalytic residues and an extended fibrinogen recognition site distinct from the catalytic site (Binnie and Lord, 1993). Thrombin binds to the central domain of the fibrinogen molecule via ionic interactions (Vali and Scheraga, 1988; Kaczmarek and McDonagh, 1988), facilitated by a cluster of positively charged residues in the thrombin molecule termed the "anion-binding exosite" (Fenton *et al*, 1988). Fibrinopeptide A release, through cleavage at Arg_{16}-Gly_{17} in the Aα fibrinogen chain, initially occurs more rapidly than release of fibrinopeptide B. This delay is thought to be necessary for normal protofibril and fibre assembly. After a lag period fibrinopeptide B release accelerates, a mechanism thought to be due to the presence of polymerized protofibrils (Ruff *et al*, 1988). Following fibrinopeptide B release, through cleavage of the Bβ chain at Arg_{14}-Gly_{15}, lateral aggregation occurs allowing for the formation of thick, well-ordered fibres (Weisel *et al*, 1993).

The thrombin release of fibrinopeptides A and B results in the formation of the intermediate fibrin monomer and exposure of new binding sites. Loss of fibrinopeptide A exposes the peptide sequence Gly-Pro-Arg (GPR) and allows calcium-dependent binding on the E domain of the fibrinogen molecule with a complementary site $\gamma_{357\text{-}373}$ (Cierniewski and Budzynski, 1993) on the outer D domain of another fibrinogen molecule (Olexa and Budzynski, 1980). Removal of fibrinopeptide B exposes a site on the E domain involving the sequence Gly-His-Arg-Pro (GHRP) which binds non-covalently to a complementary region on another fibrinogen molecule. Various other complementary regions have been proposed and include the γ chain of the distal D domain (Weisel, 1986), the C-terminal region of the Aα chain (Hasegawa and Sasaki, 1990) and the distal domain of the Bβ chain (Laudano and Doolittle, 1981). Although the GPR region in the Aα chain, exposed by thrombin release of fibrinopeptide A, acts as a very effective inhibitor of fibrin polymerization (Kawasaki *et al*, 1993; Laudano and Doolittle, 1980) the GHRP peptide, exposed during thrombin cleavage of the Bβ chain, is unable to prevent polymerization (Laudano and Doolittle, 1980 and 1981). Following release of fibrinopeptides, polymer formation is initiated by noncovalent intermolecular interactions between D and E domains (Olexa and Budzynski, 1980; Budzynski *et al*, 1983) forming double-stranded staggered and overlapping fibrils (Mosseson, 1990). Thereafter, lateral fibrin association occurs causing an increase in fibril thickness (Hewat *et al*, 1983). The C-terminal portion of the Aα-chains, which extend from the D-domain (Farrell *et al*, 1993), are important for lateral aggregation by intramolecular interaction of the Aα C-domains

(Gorkun *et al*, 1994). However, the C-terminal portions of the Aα chain are susceptible to plasmin cleavage and these free fragments are then able to compete for complementary binding to adjacent fibrin molecules, interfering with the normal polymerization process (Veklich *et al*, 1993).

Fibrin stabilization and crosslinking by factor XIII

The final step in formation of an insoluble fibrin clot involves formation of covalent isopeptide bonds (Folk *et al*, 1977), a reaction which is catalyzed by a calcium dependent enzyme, factor XIIIa or plasma transglutaminase. Isopeptide linkages are formed by replacing the γ-amide group of glutamine residues of one chain with the ε-amino group of lysine residues of another chain (Folk *et al*, 1977). Factor XIIIa may be generated from two zymogen forms of the enzyme: plasma factor XIII, an a_2b_2 tetramer that circulates freely in the plasma, and platelet factor XIII, an a_2 dimer found primarily in platelets and placenta (Hornyak and Shafer, 1992). Platelets have been shown to contain nearly 50% of blood factor XIII (McDonagh *et al*, 1969, Lopaciuk *et al*, 1976) and provide additional factor XIII at sites of vessel injury or thrombosis where they accumulate in reponse to tissue damage. A tissue transglutaminase also exists as a cytosolic enzyme present in a variety of cells. It is released into the plasma environment during haemolysis or from endothelial cells during atherosclerotic plaque formation. This tissue transglutaminase is reported to be responsible for intramolecular crosslinking between the constituent Aα and γ chains of monomeric fibrinogen, giving rise to an Aα.γ hybrid which is virtually incoagulable by thrombin (Murthy *et al*, 1991).

Plasma factor XIII is activated by the thrombin-catalyzed proteolysis at Arg_{37} in the α subunits releasing an activation peptide (Ichinose *et al*, 1986a). Exposure of the cleaved zymogen to calcium ion results in dissociation of the *b* subunits yielding catalytically active factor XIIIa (Lorand and Konishi, 1964; Chung *et el*, 1974). The zymogen form of plasma factor XIII circulates as a complex bound to the D domain of plasma fibrinogen (Greenberg and Schuman, 1982; Mary *et al*, 1987). In fibrin stabilization, several crosslinking reactions occur including formation of fibrin γ-chain dimers (Chen and Doolittle, 1971), incorporation of the plasmin inhibitor, α_2-antiplasmin, into the α-chain of fibrin (Sakata and Aoki, 1980) and the formation of fibrin α-chain polymers (McKee *et al*, 1970), which is the critical determinant in maintaining clot integrity (Gaffney and Whitaker, 1979). The C-terminal part of the Aα chain is an autonomous, functionally active and flexible region that plays a key role in α polymer formation and

functionally active and flexible region that plays a key role in α polymer formation and stabilization of fibrin clots by factor XIIIa (Mutsuka *et al*, 1996). The first two reactions are relatively rapid, occurring within 5 minutes after initiation of clot formation whereas the latter reaction may take many hours to complete (Finlayson and Aronsen, 1974). These reactions result in mechanical stabilization of the thrombus (Shen and Lorand, 1987) as well as increased resistance to plasmin mediated degradation (Gormsen *et al*, 1967). In the presence of high concentrations of factor XIII the fibrin produced exhibits greater size and complexity of highly crosslinked α-polymer chains and increased resistance to solubilization by plasmin (Francis and Marder, 1988). This increased resistance to plasmin degradation is due to multiple effects of factor XIII: decreased plasminogen binding (Sakata *et al*, 1984), increased inhibitor crosslinking (Sakata and Aoki, 1980) and the ability of factor XIIIa to crosslink both fibronectin (Mosher, 1975) and vWF (Hada *et al*, 1986) to fibrin Aα chains. In addition, rigidity and elasticity of the gel are dependent on the extent of factor XIII crosslinking (Shen *et al*, 1975; Gladner and Nossal, 1983). *In vivo* proof of the participation of factor XIII in clot rigidity was shown by the use of a factor XIIIa inhibitor, L-722,151, before initiation of thrombus formation in a canine model of coronary thrombosis. Administration of the inhibitor was associated with a threefold decrease in thrombolysis time when using tissue-type plasminogen activator (t-PA) (Shebuski *et al*, 1990).

Fibrin polymers have been found to be responsible for the fibrin(ogen)-dependent acceleration of factor XIII activation (Lewis *et al*, 1985) but this occurs only when the γ-γ dimer formation is less than 40% complete (Lewis *et al*, 1985). This ensures targeting of factor XIIIa activity at the growing fibrin protofibrils, without excessive crosslinking within the fully assembled fibrin clot. The net result of the fibrin assembly process is the formation of a three dimensional, highly interconnected network of fibrin fibres which functions as a haemostatic plug (Hantgan *et al*, 1994). Fibrin clots formed by these non-covalent interactions will, under the influence of various forces, undergo viscous deformation changes (Mosseson, 1990). However, incorporation of covalent bonds into their structure by the transglutaminase factor XIIIa dramatically changes the viscoelastic properties of the clot (Mosseson, 1990).

Plasmic digestion of fibrin(ogen)

Modification of soluble fibrinogen by thrombin to form an insoluble fibrin network is essential for wound healing and haemostasis. However, it is of paramount importance that there exists a system to allow for dismantling of this fibrin clot as wound repair progresses and to act as a

safeguard against the threat of thrombosis and embolism. A proteolytic process complementary to the one that generates thrombin is thus present and dissolution progresses primarily through a plasmin mediated pathway. Circulating plasminogen binds selectively to fibrin allowing for generation of plasmin activity at the clot surface (Nieuwenhuizen, 1988; Fears, 1989). The zymogen plasminogen, released from vascular endothelial cells, is converted to active plasmin via t-PA, a serine protease showing specificity for the Arg_{561}-Val_{562} peptide bond in plasminogen (Collen, 1980). In addition to its action in plasminogen activation recombinant t-PA is able to release fibrinopeptides A and B from fibrinogen (Weitz *et al*, 1988).

t-PA binds to the D-domain of fibrin(ogen) and two distinct CNBr fragments of fibrinogen (FCB) in this region, namely FCB-2 and FCB-3, have been found to contain amino acid sequences required to stimulate plasminogen activation by t-PA (Niewenhuizen *et al*, 1983; Schielen *et al*, 1991). Binding of t-PA to fibrin involves, for the most part, a lysine-independent interaction with the D-domain contributed by sequences present in FCB-5 and FCB-2. A lysine-dependent interaction with FCB-2 only is also detectable but is probably only of minor relevance (Grailke *et al*, 1994). Urokinase-type plasminogen activator (u-PA) is also a highly specific serine protease that cleaves plasminogen at the same site as t-PA to generate active plasmin (Linjen and Collen, 1991). It is synthesized by a variety of human cells and secreted initially as prourokinase or single-chain urokinase which is enzymatically inactive. Following cleavage of urokinase at Lys_{158}-Ile_{159} by a variety of proteases including plasmin it is converted to active two-chain urokinase (Nielsen *et al*, 1982; Ichinose *et al*, 1986b). It has been reported that t-PA, which binds strongly to fibrin, is primarily involved in fibrinolysis whereas u-PA, which does not show significant affinity for fibrin, is involved in degradation of the tissue matrix of basement membrane proteins (Thorsen *et al*, 1972; Blasi *et al*, 1987). However, Husain (1993) has shown that in the presence of zinc, single-chain u-PA is specifically and effectively bound to fibrin and therefore may have a role to play in fibrinolysis. Plasminogen and t-PA bind specifically to the fibrin clot surface (Nieuwenhuizen, 1988) and plasmin generated fragments of fibrin, D-dimer, and fibrinogen are able to accelerate plasminogen activation (Verheijen *et al*, 1982). The sites within the molecule responsible for the enhanced activation appear to reside at positions $A\alpha_{148\text{-}160}$ (FCB-2) (Vorskuilen *et al*, 1987) and $\gamma_{311\text{-}379}$ (FCB-5) (Yonekawa *et al*, 1992), portions of the $A\alpha$ and γ chain exposed during the conformational changes occurring following cleavage of fibrinopeptide A and B (Schielen *et al*, 1991). Therefore fibrin is able to regulate its own destruction. Plasminogen has been shown to accumulate severalfold in a superficial layer on clot during lysis (Sakharov and Rijken, 1995). Under conditions of diffusional transport of fibrinolytic enzymes

from outside a clot, which occurs in situations when insignificant or no pressure is applied across the thrombus (non-occluding thrombi, venous thrombus), extensive lysis appears to be restricted to a zone of 5-8 μm from the clot surface and a sharp zone of demarcation exists between completely lysed and virtually non-lysed areas of a clot (Sakharov *et al*, 1996). This allows for a layer by layer dissolution of thrombi without the release of partially lysed but insoluble material into the circulation.

Fibrinolysis appears to follow a similar course to that of plasmin mediated fibrinogenolysis (Gaffney, 1973) with remarkably similar products being generated (Gaffney, 1973; Ferguson *et al*, 1975). Thus, the intermolecular associations between the fibrin units, which are limited to the nodular bodies of the fibrin monomers, do not protect them from proteolytic attack. In addition plasmin, which shows a specificity for lysyl peptide bonds (Plow, 1980), needs only to cleave at the narrow three-stranded connectors to initiate dissolution. Thereafter, digestion may then continue until the production of a set of core products (Pizzo *et al*, 1973).

Plasmin begins its digestion of fibrinogen by attack at several sites within the Aα chain from the carboxyl terminus. This releases several Aα chain peptides termed fragments A, B and C, with molecular weights of approximately 15 kDa (Pizzo *et al*, 1973) and the fibrinogen product, fragment X (240-265 kDa) which may exist in a number of forms depending upon the extent of Aα chain and subsequent Bβ chain degradation. The γ chain of the molecule is the most resistant to degradation but as it begins to break down and the α and β chains are further cleaved, other forms of fragment X along with fragments D (83-100 kDa), E (41 kDa) and Y (approximately 150 kDa) appear. Fragments X and Y are transient intermediate products whereas A-E represent terminal products of plasmin digestion (Doolittle, 1984). Fragment D contains extensively degraded Aα chains and partially degraded Bβ and γ chains. In addition fragment D may contain different forms of the cleaved γ chain (Pizzo *et al*, 1973). Fragment E contains extensively degraded Aα, Bβ and γ chains which are linked through disulfide bonds.

The major difference between fibrinogen and fibrin digests is observed with factor XIIIa-stabilized fibrin. Factor XIII transglutaminase catalyzes a reaction in which an isopeptide linkage is formed by replacing the γ-amide group of glutamine residues of one chain with the ε-amino group of lysine residues of another chain (Schwartz *et al*, 1973). This cross-linking of two antiparallel gamma-chain regions results in plasmin digestion generating D-dimer fragments and not individual D fragments (Chen and Doolittle, 1971). In addition, interaction of the central E domain with two

complex to be isolated (Hundry-Clergeon *et al*, 1974; Gaffney *et al*, 1975). Crosslinked fibrin polymers circulating within the plasma are linked primarily through their γ chains and the plasmic degradation product of these crosslinked fibrin polymers is D-dimer (Kornberg *et al*, 1992). During fibrinolysis, plasmic digestion releases soluble products that remain complexed to active thrombin (Francis *et al*, 1983) which may be important in the phenomenon of rethrombosis following thrombolytic treatment (Mirshahi *et al*, 1989). Also of interest is that cross-linked complexes from plasmin dissolved clot that have been observed by electron microscopy reveal plasminogen bound to the end of fibrin. This allows for bridging of the ends of two fibrin molecules with the formation of larger complexes (Weisel *et al*, 1994).

The incorporation of cellular components such as platelets and leukocytes into fibrin clots may have a significant effect on the speed of subsequent clot lysis (see chapter 4). Actin filaments released from cells as a consequence of cell death may also become entrapped within a developing thrombus. Their presence, which renders the thrombus more brittle and unable to withstand substantial deformation without structural changes, is able to inhibit plasmin-mediated fibrinolysis through a change in the viscoelastic property of the clot (Janmey *et al*, 1992).

Nonplasmin-mediated fibrinolysis

The well characterized plasminogen/plasmin system has long been seen as the major component in contributing to fibrinolysis. However, it has become clear that the cellular consituents of the blood, and in particular the neutrophil, play a significant role in the fibrinolytic process. This cellular activity may be increased by various stimuli including exercise (Langleben and Moroz, 1985), venous occlusion (Hammouda and Moroz, 1986) and surgical trauma (Moroz and MacLean, 1979). It has been suggested that the success of exogenous thrombolytic therapy in producing coronary reperfusion following acute myocardial infarction is due to intrinsic cellular phase activity present before the initiation of treatment (Langleben *et al*, 1990). Rulot (1904) and Opie (1907), at the turn of the century, showed that leukocytes contain proteolytic enzymes that are capable of digesting fibrin and that the presence of leukocytes within a fibrin clot is able to significantly increase the rate of fibrinolysis. Leukocytes are found in close contact to fibrin at sites of ongoing inflammatory activity. They accumulate intravascularly, within complex thrombi consisting of fibrin and platelets (Barnhart, 1965) and more recently have been found to be incorporated into platelet-rich clots due to specific cellular adhesive processes, related to the expression of P-selectin on activated platelets binding to a counter receptor on the neutrophil membrane (Palabrica *et al*, 1992). Extravascular accumulation may also occur under

membrane (Palabrica *et al*, 1992). Extravascular accumulation may also occur under circumstances where leukocytes migrate to sites of injury associated with fibrin deposition (Riddle and Barnhart, 1964). Neutrophils express the cellular receptor for urokinase-type plasminogen activator (uPA-R). This receptor also associates in a reversible fashion with the leukocyte integrin Mac-1 (Kindzelskii *et al*, 1996). Following binding of u-PA to its receptor the enzyme becomes localized to the surface of the cell which may facilitate neutrophil infiltration into fibrin clot, enhancing fibrinolysis (Henjgers *et al*, 1995). Although it was noted in 1907 (Opie) that white blood cells can dissolve fibrin clots the majority of studies have investigated fibrinogenolysis rather than fibrinolysis by leukocytes.

In 1984 Moroz stated that "all fibrinolytic activity that is extractable from PMN (neutrophils) can be accounted for by two lysosomal enzymes, the elastase-like proteinase (PMN elastase) and the chymotrypsin-like proteinase (cathepsin G)", a view supported by many other investigators (Plow, 1980; Plow and Edgington, 1978; Schmidt and Havemann, 1977). These enzymes, present within the primary granules of the cell (Ohlsson *et al*, 1977), are capable of degrading both fibrinogen and fibrin in a manner that is distinct from that of plasmin (Plow and Edgington, 1975). Gramse *et al* (1978) looked at fibrinogen degradation by purified human neutrophil elastase, the major enzyme responsible for neutrophil-mediated fibrinogenolytic activity (Bilezikian and Nossel, 1977), and found the Aα chain to be highly susceptible to this enzyme with the γ chain much less susceptible. Degradation was progressive as well as time and concentration dependent, whereas plasminolysis gave rise to specific end products. Elastase shows specificity for alanyl peptide bonds and release of fibrinopeptide A and fibrinopeptide B containing fragments occurs early on, following its interaction with fibrinogen, whereas plasmin produces a fibrinopeptide A containing fragment only at the final stages of digestion (Bilezikian and Nossel, 1977; Plow 1980). The initial elastase-derived degradation products arise from cleavage at the C-terminus of the molecule (Bach-Gansmo *et al*, 1994) and have anticoagulant properties but unlike plasmin, cleavage is not confined to the carboxyl termini of the Aα and Bβ chains (Plow, 1980). Cathepsin G also contributes to the fibrinogenolytic and fibrinolytic action of the neutrophil (Plow, 1980) and shows specificity for leucyl bonds as well as aromatic amino acids (Zimmerman and Ashe, 1977), in contrast to plasmin which shows specificity for lysyl peptide bonds (Weinstein and Doolittle, 1972). In the presence of calcium chloride cathepsin G initially cleaves the Aα chain followed by the Bβ and then the γ chain of fibrinogen (Gramse *et al*, 1980), whereas in the absence of calcium ions the γ chain shows greater susceptibility to cathepsin G than the Bβ chain. Elastase has been shown to inactivate plasminogen activator inhibitor-1 (PAI-1) leading to enhanced fibrinolysis

and cathepsin G with limited proteolysis giving rise to miniplasminogen, which is more readily activated by plasminogen-activators (Machovich *et al*, 1990). Impaired clot lysis in the presence of neutrophil elastase has also been described due to reduced t-PA mediated activation of plasminogen (Bach-Gansmo *et al*, 1995).

Kolev *et al* (1996) investigated the relative contributions of plasmin, miniplasmin, neutrophil elastase and cathepsin G to the fibrinolytic process. Mini-plasminogen is produced following exposure of plasminogen to neutrophil elastase. Activation by plasminogen-activators yields miniplasmin (Moroz, 1981), a molecule with enhanced fibrinolytic activity compared to plasmin and relatively resistant to inhibition by α_2-antiplasmin (Moroz, 1981). When present on the surface of preformed clot elastase and cathepsin G were less efficient than plasmin and mini-plasmin at fibrin degradation. Under conditions of crosslinking of the clot by factor XIIIa in the presence of α_2-antiplasmin there is increased clot resistance to plasmin and mini-plasmin while the action of leukocyte proteases is unaffected. Both elastase and cathepsin G are able to potentiate the action of plasmin on crosslinked fibrin containing plasmin inhibitor, possibly by their conversion of plasmin to miniplasmin and inactivation of plasmin inhibitor (Kolev *et al*, 1996).

Numerous elastase inhibitors are found within plasma and interstitial spaces (Travis and Salvesan, 1983). However, Weitz *et al* (1987) were able to show that N-formyl-methionyl-leucyl-phenylalanine (FMLP)-stimulated neutrophils, migrating through fibrinogen-coated filters in the presence of proteinase inhibitors, were still able to exhibit significant elastase-mediated fibrinogenolytic activity. In contrast, these same proteinase inhibitors were able to completely block the fibrinogenolytic activity of soluble purified elastase. An explanation for this phenomenon was provided by Loike *et al* (1992). FMLP-stimulated neutrophils are able to interact with fibrinogen coated surfaces via the adhesive surface-membrane receptors, the β_2-integrins. One of the consequences of this interaction is that protected compartments are formed allowing for release of proteases into an enviroment protected from proteinase inhibitors present within the plasma. These compartments are able to exclude plasma proteins of >40 kDa and thus enable neutrophil proteases to degrade extracellular matrix components despite the presence of inhibitors. A role for oxidant inactivation of proteinase inhibitors, in particular α_1-proteinase inhibitor, has been suggested (Carp and Janoff, 1978; Matheson *et al*, 1981). In view of the fact that stimulated neutrophils are a source of oxidant activity it was proposed that they may play a role in decreasing the local elastase inhibitory capacity of α_1-proteinase inhibitor (Carp and Janoff, 1979). However, Campbell *et al* (1982) were unable to show that this was the case under

1979). However, Campbell *et al* (1982) were unable to show that this was the case under physiological conditions due to the presence of antioxidants and competetive antagonists. This remains a controversial point however, as Bangalore and Travis (1994) were able to show that upon degranulation of neutrophils stimulated by FMLP or phorbol 12-myristate 13-acetate (PMA), released elastase and cathepsin G are able to re-bind in an active form to the neutrophil membrane, in a form relatively resistant to proteinase inhibitors. In addition, myeloperoxidase is released and likewise is capable of binding to the cell membrane where it was found to oxidatively inactivate α_1-proteinase inhibitor. Owen *et al* (1995a) showed in a similar study that membrane bound elastase and cathepsin G remain catalytically active, yet relatively resistant to proteinase inhibitors.

More recently a 600kDa neutrophil membrane neutral protease has been described capable of degrading fibrinogen in a manner distinct from that of neutrophil lysosomal enzymes and plasmin (Kelly *et al*, 1994). It cleaves the bond between amino acids valine and glutamic acid at positions 21 and 22 respectively from the Aα chain to release an Aα1-21 peptide, previously seen as a reflection of overall elastase activity (Weitz *et al*, 1986). Digestion of the Bβ chain occurs at positions within the C-terminus while proteolysis of the N-terminus of the γ chain is produced at the bond between amino acids isoleucine and glycine at positions 394 and 395 (Kelly *et al*, 1994). The products thus formed do not exhibit anticoagulant activity.

Relatively little data is available on the contribution of other blood cells to fibrin(ogen)olysis. Platelets do possess proteases capable of digesting fibrin (Nachman and Ferris, 1968) but these appear to play only a minor role in the process (Moroz, 1977). Peripheral blood lymphocytes have been found to have no detectable fibrinolytic activity and erythrocytes negligible amounts while the fibrinolytic activity of eosinophils appears similar to that of neutrophils (Moroz, 1984). Monocytes do possess fibrinolytic potential although this is at a relatively low level compared to that provided by the neutrophil (Grau and Moroz, 1989). Werb and Gordon (1975) described elastase secretion by macrophages. This enzyme was immunologically unrelated to neutrophil elastase. Release of monocyte non-plasmin fibrinolytic enzymes appears to be triggered by fibrin(ogen) contact with the cell (Grau and Moroz, 1989). This interaction with the cell is mediated via the CD11b/CD18 integrin and following binding of fibrin(ogen) the ligand is internalized and degraded via a lysosomal-mediated pathway (Simon *et al*, 1993). It has now been shown that the enzyme responsible for monocyte-mediated plasminogen-independent fibrinolysis is cathepsin D. This is an aspartyl protease with a pH activity profile similar to that of neutrophil

lysosomal enzymes. Cleavage of the α, β, and γ chains by cathepsin D generates multiple low molecular weight fragments (Simon *et al*, 1994).

Bioactivity of fibrin(ogen) degradation products

Fibrin has long been recognised as an histological feature of both the acute and chronic inflammatory process. Moreover, fibrin(ogen)-derived proteolytic fragments have been found to produce many effects relevant to inflammation. High levels of FDP's are present during situations associated with ongoing thrombosis and fibrinolysis such as in patients with disseminated intravascular coagulation, commonly associated with septicaemia and eclampsia of pregnancy, and those receiving exogenous thrombolytic agents for treatment of pulmonary embolism or myocardial infarction. In particular the severity of atherosclerosis shows significant correlation with circulating fibrinogen and D-dimer levels (Lassila *et al*, 1993). The effectiveness of fibrinolytic therapy following administration for thrombosis is often monitored by the plasma levels of D-dimer. However, the D-dimer levels measured following fibrinolytic therapy may well be a result of the effect of plasmin on soluble fibrin monomer as well as lysis of solid thrombus (Francis and Kornberg, 1992). The fibrin(ogen) derived peptide $B\beta_{30-43}$ is a sensitive marker of neutrophil activation and proteolytic activity in clot lysis which occurs early in the course of acute myocardial infarction and is accelerated by fibrinolytic treatment (Sylven *et al*, 1992). The biological activity associated with these various products may have important immunomodulatory consequences in certain pathological situations. Numerous investigators have studied these effects, sometimes with conflicting results.

Wound healing and atherosclerosis are processes that involve cell proliferation which is dependent on various growth factors. Lorenzet *et al* (1992) were able to show that products generated by plasmin digestion of fibrinogen were able to increase the release of endothelial cell-derived growth factors from porcine aortic endothelial cells. This property appeared to be related to the low molecular weight FDP's (ie <10 kDa) and was not displayed by intact fibrinogen, nor fragments D and E. Along with the release of growth factors, the low molecular weight products were also able to induce endothelial cell damage and with prolonged incubations, cell detachment and death.

Human fibrinopeptide B, a 14 amino acid peptide released from the amino-terminus of the Bβ-chains of fibrinogen by thrombin and also contained within the primary plasmin cleavage product of fibrinogen, has been shown to induce neutrophil chemotaxis without release of lysosomal enzymes or production of superoxide anion (Senior *et al*, 1986; Kay *et al*, 1974). Skogen *et al*

(1988) subsequently found that $B\beta_{1-42}$, released from the Bβ chain of fibrinogen by plasmin, could act as a chemoattractant and this activity was not confined only to the Bβ chain 1-14 segment. Other properties ascribed to low molecular weight polypeptides derived from fibrinogen include inhibition of lymphocyte blastogenesis (Edgington *et al*, 1985) as well as anticoagulant activity (Lau, 1993). Weitz *et al* (1991) showed that the soluble products of t-PA-induced lysis of cross-linked fibrin are able to potentiate t-PA-mediated fibrinogenolysis by providing a surface for t-PA and plasminogen binding, and in doing so are able to promote plasmin generation.

The effects of fragment D on endothelial cell function have been extensively studied. High levels of this fragment are seen in patients with adult respiratory distress syndrome (Rinaldo and Rogers, 1982), thrombotic thrombocytopaenic purpura (Ridolfi and Bell, 1981) and disseminated intravascular coagulopathy (Bell, 1980) which are associated with endothelial cell abnormalities. Dang *et al* (1985) showed that fibrinogen fragment D was able to produce a specific disorganization of cultured vascular endothelial cells with cell retraction, detachment and redistribution of actin filaments. The mechanism for this endothelial cell detachment has been ascribed to the ability of fragment D to increase secretion of endothelial plasminogen activators which enhances the generation of plasmin and contributes to the proteolysis of extracellular matrix (Ge *et al*, 1992). The effect of both fragment D and E on neutrophil function has also been investigated. Kazura *et al* (1989) found that elevated levels of these products were able to inhibit several neutrophil functions critical to the bactericidal role of these cells. These included inhibition of superoxide release and chemotaxis in response to FMLP as well as inhibition of superoxide production on exposure to PMA and zymosan-activated serum. In addition, Fischer *et al* (1991) were able to demonstrate that at a concentration of 50 μg/ml, fragments D and E were able to inhibit attachment of neutrophils to both glass and human umbilical vein endothelial cells.

Fragment D has been reported to play a role in stimulating the proliferation of certain human haematopoietic cells (Hatzfeld *et al*, 1982). However, Robson *et al* (1993) reported that products with an intact D domain C-terminal γ chain are able to inhibit lymphocyte proliferation in response to T-cell mitogens, allogenic mononuclear leukocytes and anti-CD3 *in vitro*. Fragment DD (D-dimer) binds specifically to monocytes by the integrin CD11b/CD18 (Altieri *et al*, 1990) and following intracellular degradation the synthesis and release of biologically active IL-1β, IL-6 and plasminogen activator inhibitors from monocytes is enhanced (Robson *et al*, 1994).

Fragment E has been shown to be associated with both angiogenic and mitogenic activity in experiments using chick chorioallantoic membranes (Stirk *et al*, 1993). The key event in the

Fragment E has been shown to be associated with both angiogenic and mitogenic activity in experiments using chick chorioallantoic membranes (Stirk *et al*, 1993). The key event in the formation of stenosing atherosclerotic lesions is thought to be focal smooth-muscle proliferation (Schwartz *et al*, 1990), thus fragment E may be playing an important role in this pathological process. The crucial event in conferring biological activity to this fragment appears to be the initial cleavage of fibrinopeptides from E by thrombin (Thompson *et al*, 1992) and is therefore confined to fibrin- and not fibrinogen-derived fragment E.

Both fragment D and E have been found to exhibit anticoagulant properties. Fragment D, which is 6-fold more potent as an anticoagulant than fragment E (Marder *et al*, 1969), inhibits fibrin polymerization by binding to sites complementary to it on the E domain of fibrin monomer (Olexa *et al*, 1981) whereas fragment E acts as an anticoagulant by competitive inhibition of the action of thrombin on fibrinogen (Larrieu *et al*, 1972).

The biological effects of fibrin(ogen) degradation fragments are therefore numerous and varied and increases in the concentration of these fragments may have a profound influence on inflammatory responses.

CHAPTER 3

Neutrophils

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Neutrophils

Introduction

Neutrophils, first described by Ehrlich in 1879, represent the first line of defense against invading pathogens both within the circulation and at extravascular sites and make up 90% of the circulating granulocyes. The granulocyte series, consisting of eosinophils, basophils and neutrophils, are produced in the bone marrow at a rate of approximately 80 million/min with a lifespan of two to three days (Roitt, 1989). Following differentiation within the bone marrow into committed stem cells and myeloblasts, synthesis of the azurophil granule proteins occurs at the promyelocyte stage of development. As maturation proceeds specific granules are synthesized at both the myelocyte and metamyelocyte stage with the neutrophil acquiring its functional capabilities (Borregaard, 1988). After maturation the neutrophil will, under normal circumstances, spend up to five days within the bone marrow before release into the circulation (Borregaard, 1988).

To carry out its primary function of killing foreign pathogens the neutrophil must emigrate from the vasculature to a site of inflammation. This necessitates the attachment of the cell to the vessel wall, migration into the tissue through extracellular matrix, followed by phagocytosis or exocytosis of granule contents. These processes are designed to defend the host from invading microbes but the cell has little ability to distinguish host from foreign antigens. Thus the cell's destructive potential may, under certain circumstances, be unleashed on host as well as foreign tissue (Weiss, 1989). The neutrophil is therefore important not only in host defence, but also in the pathogenesis of certain noninfectious diseases such as adult repiratory distress syndrome, rheumatoid arthritis and reperfusion injury following myocardial infarction (Malech and Gallin, 1987).

Chemotaxis depends on a unique group of surface receptors that include both low and high affinity receptors for N-formyl peptides of bacterial origin (Snyderman, 1985), receptors for C5a and those for lipid products such as PAF and leukotriene B_4 (Baggiolini *et al*, 1991). IL-8, a potent proinflammatory cytokine, also plays a key role in recruitment and activation of neutrophils. Binding is via two distinct types of receptor. The type 1 receptor interacts exclusively

with IL-8 with high affinity whereas type 2 is able to bind a variety of chemokines (Wernette-Hammond *et al*, 1995). Occupancy of these receptors leads to shape changes and directional movement (Snyderman, 1985) with the leading edge of the neutrophil orientated in the direction of the highest concentration of chemoattractant (Cohen, 1994).

Effective killing is dependent on engulfment of the foreign particle via pseudopodia and entrapment within a phagosome. This process may be nonopsonic or opsonic dependent. Nonopsonic killing occurs following neutrophil surface receptor recognition of microbial sugar residues (Ofek *et al*, 1992). Important receptors in the opsonic process include those for immunoglobulin and complement (Leigh *et al*, 1979). Microbial killing and tissue destruction is the result of both proteolytic activity (see neutrophil proteases) and the formation of reactive oxygen intermediates, in particular superoxide anions and hydrogen peroxide. Central to this is the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex. This system converts molecular oxygen into superoxide anion by transporting an electron from NADPH. These unstable superoxide anions lead to subsequent formation of hydrogen peroxide. Hypochlorous acid, which is primarily responsible for killing bacteria within phagosomes, is formed by the action of myeloperoxidase on hydrogen peroxide. This so-called respiratory burst occurs at the cell membrane with release of toxic oxygen metabolites into both the extracellular matrix and the phagosome (Van der Valk and Herman, 1987). Efficiency of the microbicidal process is also dependent on an acid enviroment within the phagosome. This is achieved by a number of mechanisms including release of lysosomal acid hydrolases into the phagosome, generation of protons by internalized microorganisms, dissociation of carbonic acid formed through the hexose monophosphate shunt and active transport of hydrogen ions (Cohen, 1994).

This review will concentrate on the proteolytic potential of the neutrophil associated with both the granules and the cell membrane. Thereafter, adhesion will be discussed with particular emphasis on the integrin superfamily of adhesion receptors and the interaction of the neutrophil with fibrin(ogen).

Neutrophil granules

The neutrophil has been recognised as a highly sophisticated cell capable of communicating with its environment with mobilization of four types of intracellular granules and vesicles depending on the stimulus received. It has the capacity for rapidly changing the protein profile of its surface membrane and consequently its capacity for interaction with other cells and microorganisms

(Kuijpers *et al*, 1991). This follows the incorporation of intracellular membranes into the plasma membrane after cell stimulation (Kuijpers *et al*, 1991; Miller *et al*, 1987). These granule membranes contain proteins with major functional implications for an effective neutrophil response at sites of inflammation (Borregaard *et al*, 1993a). Several distinct subsets of granules exist. The two well recognized categories are the peroxidase positive (primary, azurophil) granules and the peroxidase negative (secondary, specific) granules (Bainton, 1975).

Azurophil granules represent the main source of bactericidal and proteolytic potential of the neutrophil. They contain myeloperoxidase, central to the hydrogen peroxide-halide system and necessary for oxygen dependent bactericidal activity (Klebanoff, 1970), while relatively few proteins have been identified in the membrane of azurophil granules. The azurophil granules are mobilized and exocytosed only to a limited extent during activation by inflammatory mediators and it is possible that their main function is executed only after cell disintegration (Borregaard *et al*, 1993b).

The extent of peroxidase negative granule mobilization is measured by lactoferrin and vitamin B_{12}-protein release (Spitznagel *et al*, 1974). Subsets exist within the peroxidase negative granules. 15% contain lactoferrin and a novel 25 kDa protein NGAL and 60% contain both lactoferrin, NGAL and gelatinase (Kjeldsen *et al*, 1993a). A NGAL-gelatinase complex exists in these (Kjeldsen *et al*, 1993b). There is also a subset, constituting 25% of peroxidase negative granules, which contains gelatinase but no lactoferrin or NGAL (Kjeldsen *et al*, 1993c). G-proteins, capable of receptor interaction have also been found associated with membranes of specific granules (Rotrosen *et al*, 1988). Although the matrix proteins contained within specific and gelatinase granules differ, the granule membrane appears similar (Borregaard *et al*, 1993a). These granules are more readily mobilised than those containing the gelatinase-NGAL complex, which in turn are liberated to a greater extent than NGAL or lactoferrin alone (Kjeldsen *et al*, 1993c). Release occurs in response to inflammatory mediators such as granulocyte macrophage colony stimulating factor (GM-CSF), IL-8, leukotriene B_1 (LTB_1), PAF and FMLP (Borregaard *et al*, 1993b). Differential mobilization of subpopulations of specific granules was shown by Suchard and Boxer (1993). Spreading alone was found not to be sufficient for H_2O_2 production, the major requirement being exocytosis of a distinct population of lactoferrin-containing specific granules. The delivery of cytochrome b from lactoferrin-containing specific granules to the plasma membrane during activation contributes to oxidant production in neutrophils adherent to extracellular matrix proteins.

Secretory vesicles represent a specialised form of endocytic vesicle, they contain plasma proteins not synthesized by the cell. Once formed however, they behave functionally like granules with respect to mobilization and are very sensitive to secondary mediators (Sengelov *et al*, 1993), the principal intracellular signal leading to exocytosis being an increase in cytosolic calcium ion concentration (Lew, 1989). The secretory vesicles act as a source of membrane proteins that can be readily incorporated into the plasma membrane in response to cell stimulation. They contain cytochrome b_{550}, Mac-1 (CR3), CR1 and the FMLP receptor (Borregaard *et al*, 1993b; Sengelov *et al*, 1994). Secretory vesicles are virtually completely mobilized during extravasation and are not regenerated after release. The plasma proteins released are non-toxic to the host even when exocytosed into the circulation or close to the vessel wall during diapedesis (Sengelov *et al*, 1995).

Selective mobilization of granules and secretory vesicles in response to inflammatory mediators is essential for an efficient response involving adhesion, migration and bactericidal activity of neutrophils. Degranulation is a guanosine triphosphate (GTP)-dependent process. Small GTP-binding proteins are thought to be involved in differential exocytosis of different granules. Two of these, rap1 and rap2, have been found to associate with the membrane of specific granules on the plasma membrane (Maridonneau-Parini and de Gunzburg, 1992). The granules that form first are the most dense and the least mobilizable (Sengelov *et al*, 1993). It has been suggested that mobilization is brought about by granules and secretory vesicles hooking on to the microtubule system of the cell. Following exposure to a stimulus the lighter and smaller organelles move faster when exposed to mechanical forces by the microtubule apparatus (Borregaard *et al*, 1993b).

Proteases of the neutrophil

Neutrophil proteases serve two primary functions: digestion of the basement membrane and extracellular matrix components of the host during migration of the neutrophil from the intravascular compartment to sites of inflammation and destruction of foreign pathogens. The proteases of the neutrophil responsible for these processes fall primarily into either the metalloprotease or serine protease class.

Neutrophil matrix metalloproteinases

This class of enzymes contains an essential zinc binding domain and an amino terminal domain which functions to preserve the latent state of the enzyme. The enzymes are secreted as zymogens

which undergo extracellular activation and are dependent on both calcium and zinc for their activity. The C-terminal of the enzyme is the major determinant in substrate specificity (Hirose *et al*, 1993). They are able to digest extracellular matrix and basement membrane components including collagen, glycoproteins and proteoglycans (Hirose *et al*, 1993). The neutrophil is a source of two matrix metalloproteases, gelatinase and collagenase.

Neutrophil gelatinase (MMP-9)

Neutrophils secrete a 92 kDa gelatinase (MMP-9) which is highly homologous to MMP-2, a 72 kDa gelatinase found in fibroblasts, lipocytes and bronchial epithelial cells. This enzyme is found within specific granules (Mainardi *et al*, 1991) and the expression of gelatinase mRNA appears to be controlled in a coordinate fashion together with lactoferrin (Graubert *et al*, 1993). It is released as an inactive proenzyme which becomes activated by oxygen metabolites or by serine proteinases (Woessner, 1991). Both gelatinolytic and type IV collagenase activity is exhibited (Morel *et al*, 1993) contributing to the cells' ability to extravasate from the intravascular compartment and migrate through the extracellular matrix.

Neutrophil collagenase (MMP-8)

Neutrophil collagenase is stored as a 75 kDa glycosylated protein within specific granules of the neutrophil and secretion of the inactive enzyme is triggered by a range of inflammatory mediators (Knauper *et al*, 1990). Conversion to the active enzyme can be achieved by other proteinases such as cathepsin G, mercurials and oxidative processes (Knauper *et al*, 1990) and occurs as the result of removal of 80 to 81 N-terminal amino acid residues (Knauper *et al*, 1990). Unlike fibroblast procollagenase it appears resistant to plasmin activation (Murphy *et al*, 1989). Following activation the enzyme cleaves interstitial collagens at a single peptide bond. Substrate specificity for interstitial collagen is determined by a 16 amino acid sequence within the C-terminal domain of neutrophil collagenase and maximal activity is related to the integrity of a disulfide-defined loop in this region (Hirose *et al*, 1993). Mutation of Asp_{253}, within the zinc binding locus, results in complete loss of activity of the enzyme (Hirose *et al*, 1993).

The serine proteases

This class of enzyme makes up the largest group of neutrophil proteases and includes many digestive enzymes, those involved in the complement system as well as those of the coagulation and fibrinolytic pathways. The serine proteases of the blood coagulation and complement

cascades consist of only a single domain, the catalytic unit, with a molecular weight of approximately 25 kDa and lack other regulatory or binding domains. These proteases contain an active site $serine_{195}$ residue which is esterified by the inhibitor diisopropyl fluorophosphate (DFP) resulting in loss of catalytic activity. A histidine residue is also critical for effective functioning of the enzyme (Barrett, 1980). It acts as a hydrolase and utilises the serine's hydroxyl group side chain to attack and cleave internal peptide bonds. These proteases are associated with the neutrophil azurophilic granules and show similarities to pancreatic trypsin and chymotrypsin. There is evidence to suggest that they arose from a single ancestral protein via duplications and mutations (Caughey, 1994).

Human neutrophil elastase (HNE)

Elastase is present within the azurophilic granules of the cell. The enzyme has an apparent molecular weight of 30 kDa and an isoelectric point of 5.5 to 5.9. Its specific substrates are elastin and type IV collagen and it cleaves the peptide bonds on the carboxyl side of valine and, to a lesser extent, alanine. Elastase is capable of digesting the matrix components of lungs, arteries, skin and ligaments (Janoff, 1972; Palmgren *et al*, 1991) and has been implicated in the pathogenesis of emphysema, adult respiratory distress syndrome and rheumatoid arthritis (Thomas *et al*, 1991).

The most important physiological inhibitor of HNE is α_1-proteinase inhibitor, a member of the serpin superfamily (Thomas *et al*, 1991). The serpins are found in extracellular fluids and recently a serpin-like protein, that complexes with the serine proteases elastase and cathepsin G by binding to the active site, has been identified within the cytosol of the cell. It is speculated that this protects the intracellular environment from proteolytic injury during degranulation (Thomas *et al*, 1991). Congenital deficiency of the α_1-proteinase inhibitor, with absent or decreased levels within the blood and lungs, manifests itself in pulmonary emphysema (Laurell and Eriksson, 1963). Other serine protease inhibitors such as α_2-macroglobulin and antileukoprotease are also effective against elastase. Secreted HNE has the ability to rebind to the neutrophil membrane in an active form that is relatively resistant to proteinase inhibitors (Bangalore and Travis, 1994; Owen *et al*, 1995a).

In addition to its role in digestion of extracellular matrix components elastase may have profound effects on the inflammatory response. Neutrophils generate the chemotactic cytokine IL-8 after stimulation by a variety of inflammatory mediators (Baggiolini and Clark-Lewis, 1992). Serine

proteases of the neutrophil azurophil granules, HNE, cathepsin G and proteinase-3 (PR3) are able to process IL-8 and generate forms which exhibit greater biological activity than the native molecule (Padrines *et al*, 1994). Similarly, fibronectin can be degraded into low molecular weight products that have biological activity (McDonald and Kelley, 1980).

Increased neutrophil elastase release has been shown in unstable angina pectoris and acute myocardial infarction (Dinerman *et al*, 1990) suggesting significant neutrophil activation. Leukocyte elastase is thought to contribute to reperfusion injury ie: the conversion of reversibly injured myocytes and endothelial cells to irreversibly injured cells. Use of elastase inhibitors during t-PA-induced coronary artery thrombolysis has been shown to significantly decrease the amount of myocardial injury effected on reperfusion in a dog model (Nicotini *et al*, 1991). Neutrophil infiltration into ischaemic myocardium has been found to be significantly less following reperfusion in the presence of the HNE inhibitor ICI 200,880 (Mehta *et al*, 1994). Erythrocyte damage may occur in the presence of HNE due to degradation of membrane proteins (Bykowska *et al*, 1993). Modulation of platelet function due to proteolysis by elastase of membrane glycoproteins such as glycoprotein Ib and thrombin binding sites resulting in inhibition of platelet aggregation has also been shown (Brower *et al*, 1985).

There are reports in the literature of both enhanced and decreased clot lysis in the presence of HNE. Plow (1982) showed that elastase release from neutrophils occurred in concert with, or shortly after clot formation with maximal release 20 to 40 min after clot formation. This was independent of thrombin, calcium or magnesium levels. Elastase possesses the ability to cleave and inactivate PAI-1 and thereby increase fibrinolysis (Wu *et al*, 1995). However, Bach-Gansmo *et al* (1995) have shown that HNE-mediated fibrin degradation is associated with decreased t-PA activation of plasminogen, leading to impaired clot lysis. Fibrin-bound HNE is relatively resistant to inhibition (Kolev *et al*, 1994).

Elastase has the ability to increase the expression of CD18 on neutrophils along with CD11b. This was found to affect migration but not adhesion (Woodman *et al*, 1993). Neutrophil effector function with up-regulation of the oxidative response to inflammatory stimuli also occurs (Kusner and King, 1989).

Cathepsin G

Cathepsin G is a chymotrypsin-like serine protease with an apparent molecular weight of 29 kDa which is released from the azurophilic granules of the neutrophil. It has the ability to degrade

connective tissue components collagen I, collagen II and cartilage proteoglycans and also exhibits antibiotic activity (Gabay, 1994). Its release is able to upregulate the oxidative response of neutrophils (Kusner and King, 1989). As with HNE the most important physiological inhibitor is α_1-proteinase inhibitor (Thomas *et al*, 1991). Similarly, it has has the ability to degrade erythrocyte membrane proteins (Bykowska *et al*, 1993) and process IL-8 into more biologically active fragments (Padrines *et al*, 1995).

It is able to play a pro-thrombogenic role with depression of the fibrinolytic system by releasing PAI-1 from platelets and human umbilical vein endothelial cells (Pintucci *et al*, 1992). Cathepsin G is also able to act as a strong platelet agonist (Selak *et al*, 1988) with induction of platelet aggregation, increase of cytoplasmic calcium ion concentrations and a rise in thromboxane A_2 production. Cathepsin G released in areas of close membrane-to-membrane contact is resistant to antiproteinases (Evangelista *et al*, 1991; Bangalore and Travis, 1994; Owen *et al*, 1995b). This close contact is a P-selectin mediated process (Evangelista *et al*, 1993).

Proteinase 3 (PR3)

This enzyme, present within the azurophilic granules, was first described by Baggiolini *et al* (1978) but generated very little interest compared to elastase and cathepsin G until it became evident that it had the capacity to degrade elastin and induce emphysema in a hamster model (Kao *et al*, 1988). The disease produced was of equal or greater severity than that mediated by HNE. PR3 is also able to degrade fibronectin and type IV collagen (Kao *et al*, 1988).

PR3 is a neutral serine protease of approximate molecular weight 29 kDa and is present in concentrations intermediate between that of elastase and cathepsin G, with an amino-terminal (N-terminal) amino acid sequence highly homologous to theirs (Campanelli *et al*, 1990a). PR3 has been shown to be a member of a cluster of elastase-related genes in the terminal region of the short arm of chromosome 19 (Sturrock *et al*, 1992). It shows a similar specific activity and substrate binding pocket to elastase and may share a major role in inflammation where neutrophil elastase has been implicated (Campanelli *et al*, 1990b). Its broad proteolytic activity suggests its importance in neutrophil migration through the basement membrane (Henson and Johnston, 1987) and in digesting phagocytosed microbes (Janoff, 1985). The inhibition profile is distinct from that of elastase and cathepsin G. There is no decreased activity in the presence of antileukoprotease and only weak inhibition by eglin C, with the most efficient inhibitory activity being displayed by α_1-proteinase inhibitor and α_2-macroglobulin (Rao *et al*, 1991).

PR3 has been shown to be the target antigen of anti-cytoplasmic antibodies circulating in the disease Wegener's granulomatosis (Csernok *et al*, 1990), characterized by systemic necrotising vasculitis and granuloma formation. The antibodies are able to activate neutrophils and induce respiratory burst and degranulation (Jennette and Falk, 1989). The antigen is located both within the azurophilic granules of the neutrophil and the cytoplasmic granules of monocytes with small amounts also present on the plasma membranes of both cell types. PR3 has been detected on the plasma membrane of neutrophils from patients with active ANCA-associated vasculitis as well as patients with sepsis who do not display ANCA. Both TNFα and IL-8 are able to induce translocation of PR3 from intragranular loci to the cell surface (Csernok *et al*, 1994). A mechanism thought to be important in the development of vasculitis is related to the induction of E-selectin expression on the surface of endothelial cells with subsequent neutrophil adhesion (Mayet *et al*, 1993).

PR3 is thought to play a role in neutrophil-mediated platelet activation. Renesto *et al* (1994) showed that PR3 and elastase, at concentrations ineffective by themselves, were able to potentiate platelet activation induced by cathepsin G. Cytolysis and detachment of endothelial cells occurs in the presence of PR3 along with elastase (Ballieux *et al*, 1994).

Azurocidin (cationic antimicrobial protein, human heparin binding protein 37) and azurophil granule protein 7 (AGP7)

Azurocidin and AGP7 represent approximately 15% of the acid extractable protein content of azurophil granules (Gabay *et al*, 1989) and show considerable sequence homology to elastase (Wilde *et al*, 1990). Azurocidin shows 65% sequence homology with the active site of elastase but the catalytic serine has been replaced with glycine and it is therefore unable to bind the inhibitor DFP (Wilde *et al*, 1990). AGP7 is 70% homologous with elastase at the active site but is capable of binding DFP (Wilde *et al*, 1990). The genes encoding for PR3, azurocidin and elastase are all found as a cluster on the short arm of chromosome 19 (Zimmer *et al*, 1992).

Azurocidin migrates as three dominant bands on sodium dodecyl sulphate polyacrylamide electrophoresis (SDS-PAGE) around apparent molecular weight 30 kDa. It appears to be an important mediator for recruiting monocytes to sites of inflammation (Pereira *et al*, 1990) and is also cytotoxic to certain microorganisms especially gram negative bacteria (Gabay, 1994). Azurocidin has also been found to be stored in small amounts in human platelets (Flodgaard *et al*, 1991). AGP7 is made up of four distinct glycoforms of molecular weights between 28 and 34 kDa

(Wilde *et al*, 1990). Both enzymes are insoluble at neutral pH and it has been suggested that they may be associated with the cell membrane (Jenne, 1994).

Proteases of the neutrophil membrane

Cell surface-associated enzymatic activity has been implicated as being important in certain cellular functions including cell-mediated cytotoxicity (Hatcher *et al*, 1978), the generation of biologically active peptides by polymorphonuclear leukocytes (Coblyn *et al*, 1979) and the inactivation of migration-inhibitory factors (Remold *et al*, 1978). Elastase-type proteases on the surface of human blood monocytes have also been suggested as having a possible role in amyloid formation (Lavie *et al*, 1980). Connelly *et al* (1985) described a neutrophil endopeptidase of the metalloprotease class associated with a membrane fraction from disrupted neutrophils that was able to cleave the chemotactic tripeptide FMLP, raising the possibility that it may have an important function in inflammation and chemotaxis. It is constitutively expressed on the cell membrane and abnormally high levels have been measured in patients with adult respiratory distress syndrome and septic pneumonia. Subsequent studies showed that inhibition of this neutral endopeptidase completely blocked FMLP-mediated chemotaxis (Painter *et al*, 1988).

Pontremoli *et al* (1986) described a neutral serine protease with an apparent molecular weight of 30 kDa and a pH optimum of 7.4 to 7.8. It is distinct from elastase and cathepsin G and is released into the extracellular medium upon stimulation of neutrophils with PMA at 10 ng/ml, a concentration which produces only minor discharge of secondary granules. At this concentration approximately 60% of total activity is released by 30 mins increasing to 80% and greater than 90% in 60 and 90 minutes respectively. The release of the enzyme from the neutrophil membrane appears to be almost entirely dependent on the addition of adenosine triphosphate (ATP) and is associated with phosphorylation of membrane proteins (Melloni *et al*, 1986). Inhibitor studies indicate that membrane-bound PKC activity is essential in the release of the neutral proteinase and the production of oxygen radicals. Pontremoli *et al* (1990) have subsequently shown that this protease is found at the sites of interaction of the membrane with the cytoskeleton and appears to be involved in downregulation of PKC activity following FMLP or PMA stimulation.

King *et al* (1987) described a chymotryptic enzyme, with a molecular weight of 150 to 180 kDa, situated on the neutrophil membrane and important in regulation of the membrane associated functions of cyclic adenosine monophosphate (cAMP) and oxygen generation. This calcium dependent enzyme, displaying serine protease activity, was subsequently shown to have an

apparent molecular weight of 65-70 kDa and an isoelectric point of 6.3 (King *et al*, 1991). Antibody 1-15, a murine anti-human neutrophil IgG_1 monoclonal antibody specific for the enzyme, inhibits activity related to cell activation, in particular superoxide generation and cAMP production after PMA or FMLP stimulation (King *et al*, 1986). It is able to inhibit polymorphonuclear cell degranulation and adhesive responses to FMLP but notably has no effect on cell chemotactic reponses to this peptide.

Bangalore and Travis (1994) described degranulation of human neutrophils following stimulation with micromolar concentrations of FMLP or PMA with concomitant release of human leucocyte elastase, cathepsin G and myeloperoxidase into the surrounding medium. It was found that some of these proteases were able to rebind in an active form to neutrophil plasma membranes or membrane fragments. These bound proteinases displayed increased resistance to inhibition by proteinase inhibitors. This phenomenon was again described by Owen *et al* (1995a). Priming of neutrophils by tumour necrosis factor-α (TNFα) and PAF is able to express increased amounts of surface bound cathepsin G (Owen *et al*, 1995b).

A neutral protease found in association with the neutrophil membrane and capable of degrading C-reactive protein (CRP) was first described in 1989 (Shephard *et al*). Inhibitor studies characterized this enzyme as a serine protease, maximally activated by PMA stimulation of the neutrophil. This PMA stimulation was achieved with greatest effect at a concentration of 10 ng/ml and resulted in increased association of CRP with neutrophils and maximal generation of trichloroacetic acid (TCA)-soluble CRP-peptides in the extracellular medium. Degradation products were detected within 2 minutes following CRP incubation with neutrophils. These soluble CRP-derived peptides were found to be biologically active and had the ability to suppress superoxide production from activated neutrophils. Further characterization of this protease was performed (Shephard *et al*, 1992) and established it as a probable submembrane protease localized at sites of interaction of the cytoskeleton with the membrane. It appeared to exist as an enzyme complex of 600 kDa which could be dissociated using SDS-PAGE into four discrete bands of 209, 316, 398 and 501 kDa molecular weight. Cellular kinases activated by PMA and inhibited by 1-(5-isoquinolinesulphonyl)-2-methylpiperazine (H7) and trifluoperazine (TFP) appear to be involved in upregulating the CRP-degrading activity of the neutrophil during cellular degradation. Further investigations (Kelly *et al*, 1994) showed the importance of this protease in acting as an alternative pathway to fibrinogenolysis to that mediated by plasmin and neutrophil lysosomal enzymes. It degrades fibrinogen in a time and concentration dependent manner with cleavage of

all three constituent chains of fibrinogen to produce unclottable products that do not exhibit anticoagulant activity. Release of an $A\alpha_{1-21}$ peptide occurs following cleavage of the bond between alanine and glutamic acid at positions 21 and 22 respectively from the N-terminus of the $A\alpha$ chain. Levels of this peptide have previously been used as a marker of human neutrophil elastase activity (Weitz *et al*, 1987).

Neutrophil adhesion

Adhesive interactions between cells and the extracellular matrix are crucial to differentiation of cells, maintenance of tissue architecture, wound healing, tumour metastasis and the inflammatory response. These adhesion events, which are mediated by cell-surface receptors that bind to ligands on adjacent cells or in the extracellular matrix, also regulate intracellular signal transduction pathways that control adhesion-induced changes in cell physiology. The adhesion events are mediated by transmembrane receptors that belong to a limited number of supergene families that include the integrins (Ruoslahti, 1991; Hynes, 1992), the immunoglobulin superfamily (Buck, 1992), cadherins (Takeichi, 1991) and selectins (Lasky, 1992; McEver, 1992).

Neutrophils are functionally dependent on various members of these supergene families for migration from the vasculature to sites of inflammation. The process of recruitment from the vascular lumen to tissue requires a cascade of sequential adhesion events (Butcher, 1991). The first step is often initiated by cytokines which are able to stimulate adhesion through leukocyte or endothelial cell activation. Leukocyte rolling on post-capillary venular endothelium is mediated via selectins with L-selectin on leukocytes interacting with E-, P-selectin or other carbohydrate ligands on other endothelial cells (Picker *et al*, 1991) in a process known as tethering. Shedding of L-selectin follows with an associated increase in avidity of other adhesion molecules, including the β_2 integrins and members of the immunoglobulin superfamily. Shedding of L-selectin occurs within seconds by a metalloprotease-dependent proteolytic event which may contribute significantly to the velocity of leukocyte rolling and may be physiologically important in limiting leukocyte aggregation and accumulation at sites of inflammation (Walcheck *et al*, 1996). The subsequent firm adhesion mediated by integrins and the immunoglobulin superfamily then allows for movement of neutrophils through the endothelium. Endocytosis of integrins and recycling to the leading edge of migrating neutrophils has been shown (Lawson and Maxfield, 1995), providing a gradient of adhesive strength from the front to the rear of the cell.

A full review of all the supergene families of adhesion receptors is beyond the scope of this thesis. Only the integrin family will be discussed in detail in this chapter with particular emphasis on the interaction of fibrinogen with neutrophils, an integrin mediated process.

The role of integrins

Integrins are transmembrane glycoproteins which exist as non-covalently associated heterodimers made up of an α (120-180 kDa) and β (90-110 kDa) subunit (Hynes, 1987). To date, 16 α subunits and eight β subunits have been identified that can heterodimerize to produce more than 20 different receptors (Clark and Brugge, 1995). Additional complexity is added by alternative splicing of the α and β subunits (Hynes, 1992). The integrin family is divided into classes based on the β subunit. The most widely distributed belonging to the β_1 class or very late antigens (VLA), (Hemler, 1990). Several of these were initially characterised on platelets before being recognised as belonging to an integrin superfamily. The second class are leukocyte specific and are made up of one of three α subunits that associate with a common β_2 subunit (Larson and Springer, 1990). The leukocyte specific β_2 integrins consist of $\alpha_L\beta_2$ (LFA-1), $\alpha_M\beta_2$ (Mac-1) and $\alpha_X\beta_2$ (p150,95) (Hynes, 1992). More recently a leukocyte specific leukocyte response integrin, with immunological homology to GPIIb-IIIa has been described. The cytoadhesins constitute the third class (Phillips *et al*, 1988) and include the receptor GPIIb-IIIa found on platelets (Plow *et al*, 1986), cells of megakaryocytic lineage (Keiffer *et al*, 1991) and some tumour cells (Chen *et al*, 1992). This integrin is reviewed in detail in Chapter 4.

Both integrin subunits are transmembrane glycoproteins with a single hydrophobic transmembrane segment. The cytoplasmic domains are for the most part short (<50 amino acids) except for that of the β_4 integrin (>1000 amino acids). The extracellular domains associate to form αβ heterodimers and the ligand binding pocket (Smyth *et al*, 1993) with high affinity ligand recognition requiring both α and β subunit participation (Loftus *et al*, 1994). Extensive disulfide bonding occurs in both subunits and the presence of compact folded domains renders them fairly resistant to proteolysis (Calvete *et al*, 1991). Receptor function is dependent on divalent cations and they may be required for αβ subunit association of some integrins (Kirchhofer *et al*, 1990).

The cytoplasmic domains interact indirectly with cytoskeletal components via linker proteins such as talin and α-actinin (Pavalko and Otey, 1994). β cytoplasmic domains are necessary and sufficient to target integrins to focal adhesions in a ligand-independent manner while α cytoplasmic domains regulate the specificty of the ligand dependent interaction (LaFlamme *et al*,

1994). Of interest is that different α subunits have very different cytoplasmic sequences and therefore a range of receptors for a given ligand can vary in their apparent associations with the cytoskeleton. This may lead to a range of cellular responses to a common extracellular ligand depending on the receptor engaged (Hynes, 1992). A conserved region (Arg_{733}-Lys_{742}) in the β_2 cytoplasmic domain appears to be critical for cytoskeletal association, assembly and transport to the plasma membrane of the $\alpha_L\beta_2$ (LFA-1) heterodimer. The conserved membrane proximal GFFKR motif confers stability to the αβ complex (Pardi *et al*, 1995). In the extracellular region of the α subunit of the β_2 integrins are two prominent features: the cation binding region and a 200 amino-acid insertion or I-domain (Corbi *et al*, 1987; Kaufman *et al*, 1991; Pytela, 1988). The binding sites for four distinct ligands for Mac-1 namely IC3b, intercellular adhesion molecule-1 (ICAM-1), fibrinogen and the counter-receptor for neutrophil homotypic adhesion, identified as L-selectin (Simon *et al*, 1993), have all been mapped to the I domain on the α chain of Mac-1 (Diamond *et al*, 1993) suggesting it is a major recognition site for both soluble and cellular ligands. More recently ICAM-2, a member of the immunoglobulin superfamily expressed on endothelial cells and a cell-surface ligand for LFA-1, has been found to bind to CD11b/CD18 through the I domain (Xie *et al*, 1995). ICAM-2 was also found to be the sole ICAM molecule found on the surface of platelets and may have a role to play in leukocyte/platelet interactions in inflammation and thrombosis (Diacovo *et al*, 1994). Three extracellular divalent cation-binding sites are present in the α subunit, a feature conserved among all integrins (Hynes, 1992). Michishita *et al* (1993) showed an additional fourth metal-binding site in CR3 in the I domain which is essential for ligand binding. Calreticulin, a highly conserved and ubiquitously expressed calcium-binding protein interacts with the cytoplasmic domain of the α subunit of all integrins (Rojiani *et al*, 1991) in an area immediately adjacent to the transmembrane domain as well as on the cell surface. Interaction with the protein affects the functional status of the integrin (Coppolino *et al*, 1994). Binding of calreticulin is via the GFFKR amino acid sequence on the integrin and its deletion results in expression of constitutively active integrins (O'Toole *et al*, 1994).

The ligands recognized by integrins are many and varied and fall into one of three classes: extracellular matrix proteins (collagen, fibronectin, fibrinogen, laminin, thrombospondin, vitronectin), plasma proteins (fibrinogen, factor X) and cell surface molecules ($C3b_i$, vascular cell adhesion molecule-1 (VCAM-1), ICAM-1,2,3) (Hynes, 1992). The amino acid sequence RGD represents a common recognition motif for many integrins (Ruoslahti and Pierschbacher, 1987). Another important recognition sequence is that of KQAGDV in fibrinogen bound by GPIIb-IIIa

and the leukocyte response integrin (Kloczewiak *et al*, 1984; Gresham *et al*, 1992). Various other sequences have been identified as being important in ligand binding to integrins. Synthetic peptides corresponding to these recognition sequences often do not bind with the same affinity as the native protein, suggesting that protein conformation and additional contact sites provide additional components necessary for optimal binding (Smyth *et al*, 1993). Interaction of integrin with ligand may also result in the exposure of novel binding sites, "ligand-induced binding sites", resulting in increased binding affinity (Hogg *et al*, 1993). The same integrin expressed on different cell types displays different ligand binding specificities which appears to be related to cell-type specific structural modification of integrins or association with cell-type specific lipid or protein factors (Smyth *et al*, 1993).

In addition to their role as adhesion molecules there is increasing evidence to suggest that integrins are essential for signal transduction both into and out of the cell. For the most part adhesion mediated by integrins requires activation of the receptor by specific signals. The activation of the receptor is able to induce a conformational change leading to ligand binding (Andrew *et al*, 1993). Ligand occupation of the receptor subsequently triggers a series of intracellular events. The cytoskeleton of the cell, consisting of microtubules and actin filaments (microfilaments), plays a crucial role in many aspects of cellular function. These include intracellular transport, cell motility, mitosis and meiosis, phagocytosis, adhesion and maintenance of cellular morphology. Current research strongly suggests that signalling via specific receptors is involved in regulating the state of actin polymerization, the organization and distribution of actin filaments and cell motility (Pavalko and LaRoche, 1993). Actin filaments must be anchored to the cytoplasmic face of the membrane in order to produce the force in cells necessary for performing many cytoskeletal dependent functions (Luna and Hitt, 1992).

Receptor signal transduction refers to events that take place after binding of ligand to receptor and generally results in activation of receptor associated G-proteins. G-proteins consist of three polypeptide subunits α, β and γ. The β and γ subunits are identical for all G-proteins whereas the GTP-binding α subunits are distinct and determine the nature of G-proteins. Following ligand binding to receptor the α subunit of the associated G-protein complex binds GTP and dissociates from the complex to perform its activity. The dissociated α-subunit complex activates phospholipase C and the resulting hydrolysis of phosphatidyl-inositol gives rise to water soluble inositol triphosphate and the lipid soluble diacylglycerol. Inositol triphosphate liberates calcium from internal stores and increases the concentration of cytosolic calcium. Diacylglycerol

participates in binding of PKC, a phospholipid calcium dependent kinase located in the cytosol of nonactivated cells. Diacylglycerol generated in the plasma membrane binds PKC and this translocates it from the cytosol to the plasma membrane where it becomes active (Litwack, 1992).

Although much is known about extracellular interactions between integrins and their ligands much less is understood about intracellular biochemical pathways and cellular functions regulated by integrins. Engagement and clustering of integrin receptors results in cell spreading and the formation of focal adhesions with integrins linking to intracellular cytoskeletal complexes and bundles of actin filaments (Pavalko and Otey, 1994) thereafter concentrating at sites of close approximation between cell and substrate (Burridge, 1988). These focal adhesions are at sites of end-on insertion of actin microfilaments into the plasma membrane and where certain cytoskeletal and signalling molecules concentrate (Schwartz, 1992). Both the α and β subunit cytoplasmic domains appear to be involved in the process of cell spreading and formation of focal adhesions (Ylanne *et al*, 1993).

Activation of β_2 integrins may be achieved with phorbol esters and inflammatory mediators such as TNF, C5a, PAF or FMLP. Upregulation of integrin expression may also occur in response to various growth and differentiation factors such as TNF-β, interferon-γ (IFN-γ) and retinoic acid (Smyth *et al*, 1993). This increased avidity of binding is transient and after a period of time "de-adherence" follows (Hogg *et al*, 1993). Phospholipase A_2-derived lipid mediators regulate a number of neutrophil responses which include degranulation, adhesion and Mac-1 expression (Jacobson and Schrier, 1993). Thus phospholipase A_2 appears to play an obligatory role in signal transduction pathways affecting acute inflammatory reponses.

It has been recognized that neutrophils adherent to extracellular matrix proteins react differently to physiological stimuli compared to neutrophils in suspension (Liles *et al*, 1995). TNF, a 17 kDa cytokine, is important for a variety of biological activities that regulate the immune response. It mediates a number of neutrophil effector functions by inducing neutrophil activation with degranulation and a respiratory burst. However this will only occur if the neutrophils are adherent to an extracellular matrix via specific integrins with CD11b/CD18 being of particular relevance (Nathan *et al*, 1989). Adherent neutrophils exposed to TNF show increased tyrosine phosphorylation of several proteins (Fuortes *et al*, 1993) with the β_2 integrins being implicated in the triggering of this process (Berton *et al*, 1994). A 42 kDa protein, identified as a member of the mitogen activated protein kinase family, shows the most striking increase in tyrosine phosphorylation following exposure of neutrophils to TNF (Rafiee *et al*, 1995). Neutrophil

stimulation by other agonists such as PAF, GM-CSF and FMLP is also able to induce functional reponses via tyrosine phosphorylation, and G proteins play a crucial role in the coupling of chemotactic factor receptors to this tyrosine phosphorylation (Rollet *et al*, 1994).

The urokinase-type plasminogen activator receptor is a 55 kDa GPI-linked membrane protein which is extensively glycosylated and able to promote leukocyte migration and the invasion of metastatic cells. It also represents a means of focussing and controlling a key component of the fibrinolytic system at the cell surface. Of particular interest is that it is physically associated with Mac-1 on intact neutrophil membranes (Xue *et al*, 1994). The interaction of the two receptors is reversible and correlates with cell polarization. During the process of polarization, as the cell begins to migrate the urokinase plasminogen activator-receptor (uPA-R) and Mac-1 dissociate by an as yet uncharacterized mechanism but reassociate when the cell returns to a spherical shape (Kindzelskii *et al*, 1996). Engagement of uPA-R leads to tyrosine phosphorylation and diacylglycerol formation (Bohuslav *et al*, 1995). In view of its role in migration and invasion, its function appears to be complementary to that of the integrin superfamily and its close association with Mac-1 indicates their functional cooperation.

Pavalko and LaRoche (1993) showed that activation of neutrophils by FMLP or cytokines induces an association between CD18 and α-actinin with α-actinin binding to an 18 amino acid region in the N-terminal half of the CD18 cytoplasmic domain. This interaction is weak or non-existent in resting cells and no similar association could be detected between CD18 and either talin or vinculin. In addition to the interaction of α-actinin with the amino terminal region of CD18, filamin also binds here and is thought to play an important role in cortical membrane stability and the regulation of integrin function at the leading edge of migrating cells (Sharma *et al*, 1995). The role of integrins as mechanoreceptors was shown by Wang *et al* (1993). Magnetic microsperes were coated with the RGD peptide and used in combination with an external magnetic field to exert controlled mechanical stress on the integrin receptor without changing cell shape. A significant mechanical constraint on the twisting of the integrin-bound beads was found due to cytoskeletal linkage to integrins. This mechanotransduction would be instantaneous and more rapid than any diffusion-based signalling system.

The link between actin cytoskeleton and the neutrophil membrane is an important mechanism for enhancing adhesion and transendothelial migration. The interaction peaks at five to ten minutes after stimulation and is reduced to near baseline at 30 minutes. There is particularly strong sequence homology in the amino terminal half of the cytoplasmic domain of the β-subunit of many

integrins. It is therefore possible that the mechanism is common to many different integrin subfamilies. Engagement of β_2 integrins in non-adherent neutrophils resulted in rapid and sustained increases in the F-actin content of neutrophils with concomitant significant formation of phosphatidylinositol triphosphate formation. This correlated with the cell's ability to undergo significant actin polymerization (Lofgren *et al*, 1993). Transition of CR3 from a low to a high avidity state is related to the phosphorylation of the cytoplasmic tail of CD18 on multiple residues (Rabb *et al*, 1993).

Protein phosphorylation is one of the earliest events detected in response to integrin stimulation. This was first shown in platelets (Clark *et al*, 1994) with integrins shown to regulate agonist-induced tyrosine phosphorylation. Serine-threonine kinase families such as PKC and mitogen activated protein (MAP) kinases are also activated upon integrin stimulation (Schlaepfer *et al*, 1994). MAP kinases appear to regulate, in part, neutrophil adhesion (Pillinger *et al*, 1996). There is now considerable evidence to suggest that certain GPI-linked proteins, which are only expressed at external membrane surfaces, interact physically with β_2 integrins. Thus, ligand binding to GPI-linked receptors by inflammatory mediators is able to influence the β_2 integrins which are in direct mechanical and biochemical contact with the cytoplasm of the cell (Petty and Todd, 1996). The intervening pathways and steps occurring following ligand binding to integrin receptor and resulting protein phosphorylation with involvement of G protein-coupled receptors are as yet uncharacterized.

Interaction of neutrophils with fibrin(ogen)

The close association of fibrin(ogen) with neutrophils has long been established. In 1987 Weitz *et al* showed that migration of neutrophils in response to a chemotactic stimulus across a fibrinogen-coated surface was accompanied by the release of HNE. The elastase released is protected from proteinase inhibitors in the surrounding medium by the presence of a protected pocket formed between the cell and the substrate. Wright *et al* (1988) showed that a neutrophil integrin receptor, the integrin CD11b/CD18, binds fibrinogen. The region recognized in the fibrinogen molecule lies at the C-terminal region of the γ chain (a portion not removed during conversion of fibrinogen to fibrin). Monocytes are also able to utilize CD11b/CD18 as a fibrinogen receptor (Trezzini *et al*, 1988). Subsequent to binding and internalization of the ligand, lysosomal degradation occurs. This binding requires adenosine diphosphate (ADP) stimulation and is calcium ion dependent (Simon *et al*, 1993).

Altieri *et al* (1990) showed that the plasmin-derived fragment D produced a dose-dependent inhibition of the interaction of fibrinogen with neutrophils and monocytes. This fragment lacks the RGD sequences located in the Aα chain as well as the C-terminal dodecapeptide recognition sequence of the γ chain. Thus they concluded that fibrinogen interacts with CD11b/CD18 through a novel recognition site not shared with other known integrins that function as fibrinogen receptors. The recognition motif was subsequently found to be $glycine_{190}$-$valine_{202}$ of the fibrinogen γ chain (Altieri *et al*, 1993). Induction of the fibrinogen binding capacity of CD11b/CD18 is characterized by rapid changes of cytosolic calcium concentration (Altieri *et al*, 1988). Diamond and Springer (1993) showed that binding of fibrinogen to phorbol ester-stimulated neutrophils occurs via a small subpopulation (approximately 10%) of CD11b/CD18. This population is recognized by an activation-specific antibody CBRM1/5 which binds to the α subunit of the integrin. This same subpopulation also mediates the binding of neutrophils to ICAM-1. The binding site for fibrinogen as well as iC3b and ICAM-1 is the I domain on the α chain of CD11b/CD18 (Diamond *et al*, 1993).

CD11c/CD18 has also been established as being able to function as a fibrinogen receptor on TNF-stimulated neutrophils and recognizes the Gly-Pro-Arg (GPR) sequence on the N-terminal domain of the $Aα_{17-19}$ chain of fibrinogen (Loike *et al*, 1991). The interaction between CD11/CD18 integrins with surface-bound ligands supports adhesion of phagocytes and their spreading, diapedesis and phagocytosis. Adherent neutrophils stimulated with TNF released large quantities of hydrogen peroxide but required adhesion to surfaces coated with extracellular matrix proteins for this to occur (Nathan *et al*, 1989). This may be an adaptation so that respiratory burst will occur in neutrophils that have aggregated, adhered or emigrated but not from those in the circulation exposed to cytokines that may have "spilled into" the blood from inflammatory sites. In the platelet-neutrophil interaction the $β_2$ integrin CD11c/CD18 is of importance. Fibrinogen appears to bridge GPIIb-IIIa on platelets with CD11c/CD18 on neutrophils and recognizes the Aα chain of platelet-expressed fibrinogen (Ruf and Patscheke, 1995).

Inhibition of neutrophil activation has been reported subsequent to fibrinogen binding (Higazi *et al*, 1994) with physiological levels inhibiting chemotactic activity triggered by zymosan activated serum, C5a and IL-8. Precoating of culture well surfaces with fibrinogen has also been reported to completely suppress the polystyrene-triggered release of hydrogen peroxide from neutrophils (Nathan, 1987).

It is evident that most studies to date have concentrated on interactions of neutrophils with fibrinogen via integrins and subsequent biological effects. However, very little data is available on interactions of neutrophils with insoluble crosslinked fibrin and adhesion receptors that may be involved in this process.

Leukocyte response integrin (LRI)

In 1989 Gresham *et al* described a heterodimeric receptor, termed the leukocyte response integrin (LRI), with ligand binding specificity for the Arg-Gly-Asp sequence and showing immunological cross-reactivity with GPIIb-IIIa on platelets. Isolation of LRI (Carreno *et al*, 1993) showed the receptor to have two subunits of 135 and 90 kD. A monoclonal antibody, B6H21, was developed that bound to this receptor and was able to inhibit both RGD-mediated ligand binding and extracellular matrix-stimulated phagocytosis by neutrophils (Gresham *et al*, 1989). Ligand binding could be rapidly inhibited by oxidants generated by neutrophils during release of myeloperoxidase and activation of the respiratory burst. It was hypothesized that the receptor may play an important role early in the inflammmatory response as a signal transducing molecule. Subsequently Brown *et al* (1990) showed that the antigen recognized by B6H12 was in fact a 50 kDa molecule expressed on the plasma membrane of all haematopoeitic cells. In the case of platelets and placenta this protein is associated with the β_3-like LRI. Antibodies directed against either of these molecules are able to inhibit Arg-Gly-Asp stimulation of phagocytosis. Thus the 50 kD protein, termed integrin associated protein (IAP), is able to modulate LRI function and may play a role in signal transduction for activation of phagocytes by RGD-containing adhesive proteins. The extracellular domain is thought to be important in the interaction of IAP with its associated integrins (Lindberg *et al*, 1993) and multiple membrane-spanning domains exist in the C-terminus which may be important in signal transduction. Gresham *et al* (1992) examined the ligand binding specificity of LRI by investigating its interaction with fibrinogen. The RGD sequence occurs twice in the fibrinogen α chain and the KQAGDV sequence occurs in the carboxyl-terminal region of the γ chain (Phillips *et al*, 1991; Smith *et al*, 1989; Kloczewiak *et al*, 1984), these regions being involved in fibrinogen binding to the platelet integrin GPIIb-IIIa. It was established that both the RGD as well as the γ chain peptide are ligands for LRI. In addition, when the native γ chain sequence KQAGDV was mutated to KGAGDV this was also able to act as a ligand. When neutrophils were stimulated with FMLP or immune complexes, ligand binding specificity for the sequence was markedly increased and reduced for RGD. Thus this unique amino acid sequence may represent a specific ligand for LRI and the specificity of LRI ligand

binding may be regulated by the activation state of the cell. Zhou and Brown (1993) have shown that ligation of LRI by surface-bound ligands and antibodies is able to directly activate the respiratory burst in neutrophils and monocytes, with LRI and IAP acting as a signal transduction unit. This LRI-IAP-initiated respiratory burst is independent of CD18.

CHAPTER 4

Platelets

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Introduction

Platelets originate in the bone marrow by fragmentation of the cytoplasm of mature, granular megakaryocytes and circulate in the blood as small cellular anucleate discs of between 2 and 5 μm diameter with a life span of approximately 10 days. Platelets have a normal circulating level of $250 \pm 50 \times 10^9$/l of blood. Once in the circulation they are able to react with a variety of agonists to fulfil their primary role of haemostasis and the maintenance of vascular integrity. Disruption of this finely tuned process may lead to pathological conditions of thrombosis or uncontrolled bleeding.

Platelets, in the quiescent state, have a smooth, convex outer membrane which on closer inspection contains numerous deep invaginations representing sites of communication between the exterior of the cell and the channels of the surface connected open canalicular system. A cytoskeleton consisting of filaments and microtubules exists which is important both structurally and functionally. The microtubules are able to maintain the discoid shape of the cell in the resting state. Upon activation the filaments are rearranged and polymerized allowing for extension of pseudopods and contractile processes which move the cytoplasmic granules towards the cell centre. Within the cytoplasm are α-granules, dense granules, glycogen particles and lipid droplets as well as mitochondria and remnants of the Golgi apparatus. The functional activity of the platelet in haemostasis involves four fundamental mechanisms, namely adhesion, aggregation, contraction and secretion. Following vascular endothelial cell damage, platelets adhere to exposed subendothelial structures and undergo various calcium dependent biochemical and shape changes. The contents of dense bodies and α-granules which are extruded into the canalicular system and finally into the extracellular medium include fibrinogen, calcium, seretonin and ADP and are able to recruit other platelets to the injured area.

External agonists interact with specific glycoprotein or glycolipid receptors on the platelet surface. These receptors may be of the G-protein, seven transmembrane domain type, or receptors used in adhesive interactions. These latter receptors generate messengers which are able to stimulate intracellular mobilization of calcium and degranulation of platelets, processes occurring

as a result of activation of kinase-phosphatase cascades (Berridge, 1987). Following engagement of a specific agonist to its receptor a biochemical signal is transduced across the membrane which allows for platelet activation. Among the substances that stimulate these intracellular pathways are thromboxane A_2, thrombin, norepinephrine, collagen and ADP (Lefkovits *et al*, 1995). Platelet release of ADP, thromboxane A_2 and serotonin in concert with the presence of other agonists in the microenvironment is able to trigger the recruitment and activation of surrounding platelets (Fuster and Jang, 1994). Regardless of the agonist involved, the end result is platelet shape change, aggregation, release of granule contents and induction of platelet coagulant activity (Coller, 1991). Glycoprotein IIb-IIIa plays a pivotal role in this process with fibrinogen and vWF the principal ligands acting to bridge platelets by binding between GPIIb-IIIa molecules on adjacent platelets.

The platelet glycoproteins can be classified as members of one of five gene families (Charo *et al*, 1994). The most abundant are those of the integrin family and include GPIIb-IIIa, which mediates platelet aggregation via fibrinogen binding. A second gene family expressed on platelets is the leucine-rich glycoprotein family of which GPIb-V-IX complex is a member. The GPIb-V-IX complex plays a critical role in the initial interaction of platelets with exposed subendothelium as well as aggregation under conditions of high shear stress in a process involving vWF. The selectins are represented by GMP-140 (P-selectin), a glycoprotein present within α-granules which becomes exposed on the platelet membrane after surface activation and is able to mediate interactions between platelets, endothelial cells and leukocytes. Members of the immunoglobulin and quadraspandin family are also present. This review will provide a detailed discussion of GPIIb-IIIa and P-selectin as they are directly relevant to the experimental work undertaken.

GPIIb-IIIa and platelet aggregation

The GPIIb-IIIa integrin was the first integrin to be associated with a human disease (Nurden and Caen, 1974; Phillips and Agin, 1977), the first to be biochemically purified (Jennings and Phillips, 1982), and the first to be expressed in completely recombinant form (O'Toole *et al*, 1989; Bodary *et al*, 1989). It is the most abundant of the platelet integrins with approximately 50 000 copies per cell and is present only on platelets and cells with megakaryoblastic potential (Phillips *et al*, 1991). It consists of an α (136 kDa) and a β (92 kDa) subunit. The light chain of the α subunit has a short cytoplasmic tail, a transmembrane region and short extracellular domain while the heavy chain is entirely extracellular (Poncz *et al*, 1987). The β subunit consists of a 762 amino

acid single polypeptide with a short cytoplasmic tail, a transmembrane region and a large extracellular domain (Fitzgerald *et al*, 1987; Phillips *et al*, 1988). The noncovalently bound receptor complex is present as a calcium-dependent heterodimer (Kunicki *et al*, 1981; Fujimura and Phillips, 1983). The cytoplasmic domains of these transmembrane glycoproteins associate with the underlying cytoplasmic actin filaments (Fox, 1985). Following ligand binding to GPIIb-IIIa cytoskeletal-integrin complexes are formed. This stabilizes the integrin-ligand interaction required for selective movement of ligand-occupied integrin into the surface connected open canalicular system (Fox, 1996).

Integrins are capable of signalling in both directions across the plasma membrane. When platelets are activated by various agonists the conformation of GPIIb-IIIa changes so that it can bind one of four soluble adhesive proteins: fibrinogen, vWF, fibronectin and vitronectin (Plow and Ginsberg, 1989, Sims *et al*, 1991). In resting platelets this integrin is able to bind fibrinogen to a limited extent which is important in the constitutive transport and incorporation of the molecule into α-granules or when fibrinogen is bound to a surface or to another activated platelet (Keiffer *et al*, 1992; Luscher and Weber, 1993). The exact involvement of this receptor in the mechanism of platelet activation remains unclear but seems to involve the cytoplasmic domain of both subunits (O'Toole *et al*, 1994) and phosphorylation of either GPIIIa (Elmore *et al*, 1990) or other molecules interacting with the integrin (Clemetson, 1995). Recently Law *et al* (1996) showed that the β_3 subunit undergoes tyrosine phosphorylation in response to thrombin-induced platelet aggregation. It appears that this tyrosine phosphorylation is important in initiating "outside-in" signalling cascades and induces the association of signalling components directly with the integrin. The phosphorylation state of the β_3 subunit represents a major factor in the exposure of ligand-binding sites on GPIIb-IIIa (van Willigen *et al*, 1996)

Activation of GPIIb-IIIa and binding of fibrinogen represent the initiation of processes leading to platelet aggregation and kinase activation with subsequent clustering of the platelet receptors (Isenberg *et al*, 1987). These clustered GPIIb-IIIa molecules occupied by fibrinogen are important in establishing links between platelets and thus allowing for aggregation to occur. In addition these clustered and occupied receptors are able to bind and activate resting platelets (Luscher and Weber, 1993). Adhesion of platelets to an altered surface such as damaged vascular endothelium is the first step in their response to vascular injury. There is a transition from a resting to an activated state in response to agonists generated subsequent to injury. Formation of a thrombus capable of arresting haemorrhage involves the interaction of platelets with one another after initial

adhesion to a thrombogenic substrate. The process of platelet aggregation only will be discussed in more detail as discussion of adhesion is beyond the scope of this review.

The mechanism of platelet aggregation following adhesion of platelets to exposed collagen depends upon the level of shear stress within the system. Under conditions of high-shear stress the receptor GPIb-IX-V complex and vWF that binds to it are central to mediating platelet aggregation (Ikeda *et al*, 1991). The importance of this interaction under high shear stress is thought to be due to the ability of vWF to take on an extended filament shape and offer an array of interactive sites capable of multivalent binding to receptors on the platelet membrane (Federici *et al*, 1989). This allows for the increased strength of interaction necessary at high shear stress. Binding is thought to increase the transmembrane flux of calcium ions, with intracellular calcium concentrations rising by two to threefold. Thus activation of the platelet may occur without the presence of agonists necessary for activation at low shear stress (Ruggeri, 1994). After initial binding to GPIb and a rise in intracellular calcium a change in the ligand specificity of GPIIb-IIIa is thought to occur that enables it to bind vWF and thereafter support platelet adhesion (Ikeda *et al*, 1993).

At lower shear stresses fibrinogen plays a vital role in platelet aggregation, acting as a molecular bridge from platelet to platelet via binding to the platelet fibrinogen receptor GPIIb-IIIa. For the most part fibrinogen will bind only to these receptors on platelets activated by various agonists. These may be strong agonists such as thrombin or thromboxane A_2 which are associated with phospholipase C and PKC activation (Shattil and Brass, 1987) or weak agonists such as ADP which result in calcium mobilization and phosphorylation of myosin light chain (Gachet and Cazenave, 1991). Exposure of platelet fibrinogen receptors may also occur subsequent to interaction with various proteolytic enzymes such as chymotrypsin and human neutrophil elastase (Niewiarowski *et al*, 1981; Kornecki *et al*, 1988). The role of neutrophil-derived proteinases in inducing platelet activation was further investigated by Renesto and Chignard (1993) who looked at the role of elastase and cathepsin G which are released simulataneously from the azurophilic granules of activated neutrophils. They found that although elastase was unable to activate platelets by itself, it was able to significantly enhance cathepsin G-induced platelet activation in terms of aggregation, dense and α-granule secretion and thromboxane B_2 production.

The interaction of fibrinogen with GPIIb-IIIa, the crucial event leading to platelet aggregation, requires recognition of specific peptide sequences contained within the fibrinogen molecule. The Arg-Gly-Asp (RGD) sequence is recognised by several integrins (Ruoslahti and Pierschbacher,

1986; D'Souza *et al*, 1991) and the fibrinogen molecule contains two RGD sequences: the 95-97 and 572-574 residues on the Aα chain, with the more N-terminal sequence primarily involved in binding to GPIIb-IIIa (Smith *et al*, 1989). Peptides containing the RGD sequence can act as potent inhibitors of the interaction between fibrinogen and GPIIb-IIIa (Plow *et al*, 1987). In contrast, other reports of binding of RGD peptides to GPIIb-IIIa, which do not require agonist activation of the platelet, show an associated conformational change within the receptor leading to the acquisition of a high affinity fibrinogen-binding state (Du *et al*, 1991). Thus, these peptides may act as partial agonists as well as competitive antagonists of integrin function. The dodecapeptide sequence 400-411 found at the extreme C-terminus of the fibrinogen γ chain (Lys-Gln-Ala-Gly-Asp-Val) and found only in fibrinogen also has the ability to bind to GPIIb-IIIa (Kloczewiak *et al*, 1982) and is probably the predominant site of fibrinogen binding (Farrell *et al*, 1992; Weisel *et al*, 1992) although this is disputed by Hantgan *et al* (1992). Both the dodecapeptide and the RGD peptide are able to induce changes in the dynamics and fluidity of the platelet membrane. Rigidification of the membrane lipid bilayer and increasing immobilization of platelet membrane proteins occurs following binding of the RGD peptide. In contrast, subsequent to interaction with the dodecapeptide, fluidization of the platelet lipid bilayer occurs with increasing mobility of thiol-containing domains of membrane proteins (Watala, 1996). Synthetic peptides containing the fibrinogen 400-411 dodecapeptide adhesion sequence are also effective inhibitors of platelet aggregation and binding of adhesive ligands. This appears to be true for both static conditions as well as in flowing whole blood (Hantgan *et al*, 1992; Bennet *et al*, 1988). Savage *et al* (1995) further investigated differences in fibrinogen binding depending on flow state and suggested that multiple sites are responsible for the adhesive potential of fibrinogen, depending on the state of receptor activation and flow conditions. The interaction of fibrinogen with its platelet receptor results in conformational changes within the fibrinogen molecule itself. The bound molecule expresses receptor induced binding sites which are not available in the free ligand. One such sequence is at $\gamma_{112\text{-}119}$ and the other is the RGD sequence at $A\alpha_{95\text{-}98}$ (Ugarova *et al*, 1993). Thus this RDG sequence does not participate in the initial binding of the molecule to GPIIb-IIIa. Ugarova *et al* (1993) proposed a model in which binding of fibrinogen to its receptor would alter the C-terminal aspects of the Aα-chains and thereafter expose sequences occurring in the coiled-coil segment between the D and E domains. These receptor-induced binding sites may then mediate unique functions of the receptor-bound molecule. In contrast to the calcium dependent binding described above, an irreversible calcium independent and non-covalent binding of immobilised fibrinogen to the purified GPIIb-IIIa receptor has been described involving novel

binding sites on both the fibrinogen molecule and the receptor (Parise *et al*, 1993). In a study investigating binding of fibrinogen degradation products to platelets, Peerschke and Galanakis (1996) showed that non-RGD, non-dodecapeptide containing platelet recognition sequences in the D and E domains of fibrinogen were able to support platelet adhesion in both a cation dependent and independent fashion.

Muller *et al* (1993) described biphasic binding of soluble fibrinogen as initially a weak and reversible interaction followed by strong and irreversible binding. More recently Wencel-Drake *et al* (1996) have shown that under conditions of irreversible binding of fibrinogen to GPIIb-IIIa, fibrinogen is rapidly internalized by the activated platelets to a surface inaccessible, intracellular pool. Following internalization there is a loss in ability of platelets to aggregate. This downregulation of surface fibrinogen in circulating platelets may represent an important antithrombotic mechanism. The possibility exists that internalized fibrinogen may be recycled back to the platelet surface in response to secondary stimulation (Wencel-Drake, 1996).

The complementary binding sites on the GPIIb-IIIa receptor have been characterized. Two RGD binding sites have been identified on the β_3 chain (IIIa): residues 119-130 (Takada *et al*, 1992) and residues 211-222 (Bajt *et al*, 1992). The sites of RGD-binding on GPIIIa are well conserved amongst other β-subunits of the integrin family (Ruoslahti, 1988). The dodecapeptide from the extreme C-terminus of the γ-chain of fibrinogen appears to selectively crosslink to GPIIb between residues 294 and 314 (D'Souza *et al*, 1990), an area that encompasses one of the putative metal-binding domains of GPIIb. Thus ligand binding may modulate calcium binding to this receptor. NMR studies have now shown that important sequences for GPIIb-IIIa interaction with fibrinogen are RHDLL and PLYM from GPIIb $_{300\text{-}314}$,HYMR from GPIIb $_{656\text{-}667}$ and SRNR from GPIIIa $_{211\text{-}223}$ (Yao and Mayo, 1996). GPIIb $_{656\text{-}667}$ is able to bind soluble fibrinogen calcium-independently and is selective for the γ chain (Calvete, 1993). Alemany *et al* (1996) showed that the sequence 274-368 in the β_3-subunit of GPIIb-IIIa acts as a calcium independent fibrinogen ligand-binding domain for the γ-chain of fibrinogen. This is distinct from the RGD-binding site, is involved in both the adhesion and aggregation of platelets and functions independently of platelet activation.

The effect of plasmin on GPIIb-IIIa may be a subject of particular importance in those patients receiving high doses of exogenous plasminogen activators for various thrombotic disorders. The effect of plasmin on platelet function is somewhat controversial with both pro- and antiaggregatory effects being reported (Niewiarowski *et al*, 1973; Shaefer and Adelman, 1985,

Torr *et al*, 1990). Pasche *et al* (1994) have also reported that plasmin is able to modify GPIIIa by a unique proteolytic event in plasma that is dependent on fibrinogen binding and thereafter is accompanied by significant reductions in fibrinogen binding and aggregation responses. This is in contrast to work done by Gouin *et al* (1991) who reported that incubation of human platelets in citrated plasma with streptokinase led to an aggregation defect related to a decrease in fibrinogen concentration and the impeding effects of fibrinogen degradation products binding to the platelet. However, there was no alteration or proteolysis of the platelet receptor GPIIb-IIIa.

Platelets and fibrinolysis

It has been found that platelet-rich clots are relatively resistant to plasmic lysis (Falk, 1992). A number of mechanisms have been proposed to account for this including platelet secretion of PAI-1 (Levi *et al*, 1992; Braaten *et al*, 1993), platelet-mediated clot retraction (Kunitada *et al*, 1992), release of α_2-plasmin inhibitor from platelet α-granules (Mullertz and Clemmensen, 1976; Sakata and Aoki, 1980), release of factor XIII from platelets leading to increased cross-linking of fibrin clot as well as cross-linking of α_2-antiplasmin to fibrin clot (Reed *et al*, 1991; Francis and Marder, 1987). However, the relative contribution of each of these processes to resistance to plasmin-mediated fibrinolysis remains controversial.

Platelets are a potent source of plasminogen activator inhibitors. They contain PAI-1 as well as a low concentration of α_2-antiplasmin (6 ng/10^8 platelets) (Plow and Collen, 1981; Booth *et al*, 1988). Investigations have tended to favour the concept that PAI-1 is the major platelet-secreted protein that is responsible for thrombolytic resistance (Braaten *et al*, 1993). However, others have found that this platelet PAI-1 is largely inactive (Booth *et al*, 1988) and argue that α_2-antiplasmin, which crosslinks to the fibrin clot by factor XIII, is a much more potent plasmic inhibitor although present in lower concentrations (Reed *et al*, 1991). Fay *et al* (1994) performed experiments with PAI-1-deficient platelets and plasminogen activators with differential sensitivity to inhibition by PAI-1 and obtained results which suggested that PAI-1 is indeed an important determinant of platelet dependent clot lysis inhibition. However, this alone could not account for the inhibition observed and it was suggested that clot retraction may be an additional important mechanism of inhibition of clot lysis as described by Kunitada *et al* (1992).

As mentioned previously, the interaction between GPIIb-IIIa and fibrinogen involves the RGD sequence as well as the KQAGDV sequence found at the C-terminus of the γ chain of fibrinogen. Braaten *et al* (1994) investigated the effect of the peptide D-RGDW, a paticularly potent

antiadhesive peptide, on platelet-delayed lysis. This peptide as well as an anti-GPIIbIIIa monoclonal antibody were able to effectively inhibit clot retraction but did not affect the overall delay in fibrinolysis due to platelets. The use of a PAI-1 resistant mutant of t-PA, with or without D-RGDW-inhibited clot retraction, could effectively increase the rate of fibrinolysis. This confirmed their earlier work implicating PAI-1 as playing an important role in thrombolytic resistance (Braaten *et al*, 1993). Of additional interest was the finding that platelet-bound fibrin exhibited a different course of fibrinolysis from that of the bulk clot. The platelet-bound fibrin took longer to lyse than the thrombus as a whole but when D-RGDW was added, acting as an agent which uncoupled fibrin from the platelet receptors, the time required to achieve 50% surface lysis decreased to a value comparable to that of the whole clot. Thus two complementary effects due to fibrin binding and clot retraction were postulated (Braaten *et al*, 1994) that could be contributing to the mechanism for lytic resistance of platelet-associated fibrin. Binding to GPIIb-IIIa and retraction creates local areas of high fibrin concentration which may in addition retard the diffusion of fibrinolytic enzymes through the matrix. The process of retraction may also exclude plasminogen and plasminogen activator containing fluid from the thrombus.

Alternatively it has been proposed that the platelet may serve as a site for assembly of proteins of the plasminogen activator system and once bound to the surface, t-PA is able to exhibit enhanced catalytic activity (Loscalzo *et al*, 1995). At high concentrations plasmin is also able to activate platelets directly, further facilitating effective binding of plasminogen and plasminogen activators and subsequent plasmin generation (Loscalzo *et al*, 1995).

Clot retraction

Clot retraction represents another important aspect of platelet function. Once the fibrin network is formed, the incorporated platelets are able to exert force on the network strands. Fibrin strands conform to the platelet surface (Morgenstern *et al*, 1984) as platelet pseudopods extend outward along fibrin bundles (Cohen *et al*, 1982). Platelets are able to constrict through contraction of microfilaments and fibrin strands are pulled into alignment (Chao *et al*, 1976). As forces develop the clot begins to retract, shrink in size and express contained serum. This process may be important in allowing for recanalization of an obstructed blood vessel. Its importance also lies in assisting with approximation of the edges of the wound and concentrating the clot in the injured area. The platelet-fibrin network subsequently serves as the scaffolding for tissue repair. The structure of the fibrin network is critical to the retraction process and in particular the fibrin fibre diameter. The fibre diameter correlates inversely with thrombin concentration (Carr and Hermans,

1978) and the thinner the fibrin fibre the greater the inhibition to clot retraction (Taylor and Muller-Eberland, 1970). Cross-linking of the clot by factor XIII is also critical to development of tension (Cohen *et al*, 1982).

The recognition sites on fibrinogen for the platelet integrin GPIIb-IIIa involve the dodecapeptide on the C-terminus of the fibrinogen γ chain and the tripeptide RGD which occurs in two places on the fibrinogen α chain (Kloczewiack *et al*, 1982; Plow *et al*, 1987). Cohen *et al* (1989) looked at the effect of peptides and monoclonal antibodies that bind to the platelet receptor GPIIb-IIIa on the development of clot tension. Peptides incorporating these recognition sites were able to increase clot tension considerably and morphological studies revealed that these peptides also increased confluence of orientated fibrin and platelet aggregates. Monoclonal antibodies directed against different epitopes on the GPIIb-IIIa complex had varying effects on clot tension, some inhibiting while others increased it. This effect of peptides on clot tension most probably occurs through increasing the interaction between platelets and polymerizing fibrin. From these studies with fibrinogen peptides and monoclonal antibodies to regions of the GPIIb-IIIa receptor Cohen *et al* (1989) concluded that clot tension requires a platelet receptor domain for polymerizing fibrin which is different from the fibrinogen receptor domain required for aggregation. Rooney *et al* (1996) constructed a recombinant human fibrinogen that lacks the γ chain four C-terminal residues which was unable to support platelet aggregation. However, clot retraction indistinguishable from that supported by normal recombinant or plasma fibrinogen could still occur. This suggests that the site on fibrinogen required for platelet aggregation is different from the site on fibrin required for clot retraction.

Following binding, GPIIb-IIIa localizes at focal adhesion sites and becomes anchored to the cytoskeleton, a key process in the initiation of cell spreading, clot retraction and signal transduction (Juliano and Haskill, 1993; Ylanne *et al*, 1993). There appears to be an important role for non-receptor tyrosine kinases in promoting integrin-cytoskeletal interactions. Subsequent to ligand-binding, cytoskeletal recruitment and/or activation of these non-receptor tyrosine kinases, such as $pp60^{c\text{-}src}$ and $pp125^{FAK}$, occurs (Oda *et al*, 1992). Thereafter these enzymes phosphorylate specific structural proteins required for the attachment of integrins to the actin-rich cytoskeleton. Schoenwaelder *et al* (1994) showed that regulation of cytoskeletal attachment of GPIIb-IIIa by platelet tyrosine kinases was an essential process for the transmission of cellular contractile forces to fibrin polymers. Of interest is that nucleated cells such as fibroblasts and tumour cells are also able to interact with the fibrin substrate and induce retraction of fibrin clots.

These cells do not possess the GPIIb-IIIa receptor but rather the β_3 integrin, $\alpha_v\beta_3$, which mediates the interaction between the fibrin substrate and the nucleated cells (Katagiri *et al*, 1995).

Neutrophil-platelet adhesion mechanisms: the role of P-selectin

The selectin family of adhesion molecules consists of three members with a standard nomenclature designating each family member according to the cell type on which it was first identified: E-selectin (endothelium), P-selectin (platelets) and L-selectin (lymphocytes) (Bevilacqua *et al*, 1991). A high degree of homology exists between these members and the genes for all three are clustered over a short region of chromosome 1 (Pigott and Power, 1993) suggesting that they arose by duplication of an ancestral gene. Each of these molecules contains an N-terminal lectin-like domain, an epidermal growth factor (EGF) repeat and a varying number of repeats of a domain found in complement regulatory proteins (Bevilacqua and Nelson, 1993). A transmembrane region is followed by a short cytoplasmic tail.

P-selectin was first discovered by investigators interested in events associated with platelet activation. A transmembrane glycoprotein of approximately 140 kD was discovered that was associated with the α-granules in resting platelets and upon activation was rapidly redistributed to the surface of the cell. This same molecule was also found in the Weibel-Palade bodies of endothelial cells and similarly could be very rapidly expressed on the cell surface following stimulation by thrombin or other agonists (Hsu-Lin *et al*, 1984; Bonfanti *et al*, 1989; McEver *et al*, 1989). It is synthesized constitutively and stored intracellularly in both platelets and endothelial cells, being targeted to secretory granules by a sorting signal present within its cytoplasmic domain (Disdier *et al*, 1992). Platelet activation is associated with phosphorylation of this region (Crovello *et al*, 1993). Following stimulation of platelets by agonists such as thrombin, histamine, terminal complement compounds and hydrogen peroxide there is rapid expression of P-selectin on the platelet surface. In endothelial cells this is followed by reinternalization, with surface expression declining within minutes (Zimmerman *et al*, 1992). Within the cytoplasmic domain a sequence exists which routes it for degradation in the lysosomes following internalization, a constitutive mechanism of down-regulation that does not require extracellular signalling (Green *et al*, 1994). The primary function of P-selectin on platelets appears to be to mediate their binding to multiple leukocyte types, while endothelial P-selectin not only plays a role in lymphocyte homing but also supports leukocyte adhesion during inflammatory processes (Hamburger and McEver, 1990; Toothill *et al*, 1990; Picker and Butcher, 1992). Following tissue injury leukocytes roll along the vessel wall, this process being mediated by P-selectin (Lawrence and Springer, 1991;

Dore *et al*, 1993), before other adhesion molecules such as integrins and receptor members of the immunoglobulin superfamily immobilize leukocytes in the area of tissue injury. Under conditions of flow, subendothelial matrix alone is unable to support neutrophil adhesion and neutrophils will adhere predominantly to platelets adherent to subendothelial matrix via P-selectin (Kuijper *et al*, 1996).

P-selectin may also be released from surface membranes following its expression on activated platelets and circulate in the plasma in a soluble and potentially functional form (Dunlop *et al*, 1992). Cloning data showed a form of P-selectin that lacks the transmembrane domain (Johnston *et al*, 1989) and in support of this human platelets have been found to contain approximately equal amounts of mRNA encoding for P-selectin with and without the transmembrane domain (Johnston *et al*, 1990). This soluble form of P-selectin may serve an anti-inflammatory function as exposure of TNF-α-activated neutrophils to the fluid-phase variant is able to inhibit neutrophil CD18-dependent adhesion to resting endothelium (Gamble *et al*, 1990) and superoxide production (Wong *et al*, 1991). Following activation of platelets by strong agonists, release of vesicles or microparticles from the platelet occurs. These microparticles, which contain GPIIb-IIIa and P-selectin, are able to adhere to fibrin and appear to play a procoagulant role (Siljander *et al*, 1996).

Two high affinity binding sites for calcium exist on P selectin (Geng *et al*, 1991) and binding of calcium induces conformational changes to the protein in a manner that is essential for leukocyte recognition. The specific ligands for selectins are carbohydrate-containing compounds notably the sialyl Le^x antigen (Bevilacqua and Nelson, 1993). Although sialyl Le^x is required for P-selectin interaction on cell surfaces, it is not sufficient for high affinity binding and a protein component within the ligand is also required for high affinity binding (Moore *et al*, 1991). The lectin domain plays a critical role in P-selectin ligand recognition, with the EGF domain conferring certain binding specificities to the adjacent lectin domain (Furie and Furie, 1995). The P-selectin ligand has been found to be distributed on myeloid cells, in particular neutrophils and monocytes, a small subpopulation of T-lymphocytes and certain malignant cells (Larsen *et al*, 1989; Moore and Thompson, 1990; Stone and Wagner, 1993). This P-selectin glycoprotein ligand-1 (PSGL-1) is a mucin-like 110 kD integral membrane protein with an extracellular, a transmembrane and a cytoplasmic domain with 50% carbohydrate component. The interaction of P-selectin with the ligand is calcium-ion dependent (Sako *et al*, 1993). Activation of neutrophils induces changes and surface expression of the P-selectin ligand with translocation and clustering of the ligand at the

uropod. Subsequent to this clustering, bound neutrophils detach from the activated platelets. This redistribution of the P-selectin ligand therefore has repercussions regarding adhesive interactions between neutrophils and activated platelets (Dore *et al*, 1996). The leukocyte integrin CD11b/CD18 displays a similar redistribution following neutrophil activation (Hughes *et al*, 1992). Thus, a possible association between CD11b/CD18 and P-selectin may exist. This theory is further supported by data showing that P-selectin has a role in activating CD18-dependent phagocytosis of unopsonized zymosan (Cooper *et al*, 1994). An additional P-selectin ligand, a 240 kD sialoglycoprotein from leukocyte membranes, has been described by Ma *et al* (1994).

The interaction of neutrophils and platelets can thus be seen to be the result of specific cellular adhesive processes in areas of thrombus formation and fibrin deposition and not simply due to mechanical trapping. Palabrica *et al* (1992) showed that the presence of leukocytes within thrombi was mediated by P-selectin expressed on activated platelets and the presence of these leukocytes promoted fibrin deposition. The mechanism for enhanced fibrin deposition is not entirely clear but Celi *et al* (1994) showed that the presence of P-selectin was able to upregulate the expression of tissue factor, the initiator of coagulation, on monocytes. Monoclonal antibodies directed against P-selectin are able to dramatically reduce the fibrin content of a developing thrombus and in turn lysis is far more rapid upon initiation of thrombolytic therapy (Toombs *et al*, 1995). Experiments to investigate the interaction of activated platelets with neutrophils were done under physiologic shear conditions in an effort to simulate a vessel wall injury (Yeo *et al*, 1994). The binding of neutrophils to activated platelets was found to be P-selectin dependent in a saturable, time and cation dependent manner. A secondary step involving neutrophil activation after platelet binding was necessary for irreversible adhesion to occur, with associated upregulation of CD11b/CD18 and downregulation of L-selectin. Nagata *et al* (1993) were able to show enhanced superoxide anion production by neutrophils following binding to activated platelets, a process that could be inhibited by the addition of anti P-selectin antibodies. Further proof that binding to P-selectin is able to alter neutrophil function was provided by Cooper *et al* (1994) who showed that following binding of neutrophils to purified, recombinant P-selectin, there was enhanced phagocytosis of unopsonized zymosan particles. The use of antibodies to either CD18 or CD11b could inhibit this phagocytosis, suggesting a signalling role for P-selectin in influencing β_2-integrin function. Subsequent to P-selectin mediated platelet adhesion to neutrophils there is inhibition of neutrophil-platelet adhesion and dissociation of existing neutrophil-platelet conjugates within 30 to 60 minutes (Rinder *et al*, 1994). Lorant *et al* (1993) looked at neutrophils adhering to endothelium via P-selectin and showed that they were upregulated and primed for enhanced

degranulation when subsequently stimulated with chemotactic factors. However, P-selectin did not induce these responses directly and the priming event appeared to be mediated by the signalling molecule, PAF.

Platelet-neutrophil interactions

The adhesion of platelets to the subendothelial matrix of a damaged vessel wall and subsequent activation has been seen as the fundamental event in the pathogenesis of thrombosis. However, it has long been realised that leukocytes are found together with platelets within both mature and developing thrombi. This is relevant not only at the site of developing thrombus but also at other sites of inflammatory reactions. Traditionally, various facets of the immune response have been studied in isolation but in reality the different components undergo complex interactions. The interaction of platelets with neutrophils is of particular relevance to this thesis.

Platelets release metabolites of arachidonic acid, PAF, platelet-derived growth factor (PDGF) and platelet factor 4 (PF4), all of which have the potential to affect various neutrophil functions including adhesion, chemotactic activity, secretion and superoxide anion generation (Weksler, 1989; Aziz *et al*, 1995). Activated platelets have been shown to adhere to neutrophils and their released products contribute to leukocyte accumulation within the fibrin clot (Larson *et al*, 1989). This stimulus of neutrophils by platelets requires intercellular contact, with fibrinogen exposure on the platelet surface (Ruf *et al*, 1992). Activated leukocytes in turn have the ability to modify platelet function. The interaction of platelets with neutrophils is mediated by specific cellular adhesive processes involving P-selectin, expressed on activated platelets, and its counter-receptor on the neutrophil.

Eicosanoids is a collective name for unsaturated lipids derived from arachidonic acid and includes prostaglandins, thromboxanes, leukotrienes, lipoxins and various hydroxy- and hydroperoxy-fatty acids that are produced by platelets. They are able to contribute to the recruitment of neutrophils to sites of vascular injury (Fretland *et al*, 1989 and 1990). In addition, stimulation of leukotriene B_4 synthesis by neutrophils occurs (Maclouf *et al*, 1982) with this molecule acting as a powerful chemoattractant (Ford-Hutchison *et al*, 1980) further amplifiying neutrophil recruitment. Transcellular biosynthesis of arachidonic acid metabolites may occur as a form of cell-cell communication enabling platelets and neutrophils to amplify the effects of their own activation (Maclouf *et al*, 1989). PDGF, PF4, serotonin and thromboxane A_2 are able to enhance neutrophil adhesion to the vessel wall (Morley and Feuerstein, 1989). While ATP and ADP, stored in platelet

dense bodies and released upon platelet activation, may produce neutrophil activation with respiratory burst (Ward *et al*, 1988) and phagocytosis (Sakamoto and Firkin, 1984). Adenine nucleotides, PDGF and other platelet-derived products are able to induce neutrophil degranulation (Del Maschio *et al*, 1989).

Besides their role in activating neutrophils, platelets may also inhibit neutrophil function. Under conditions of close neutrophil-platelet contact, platelets are able to significantly decrease elastase secretion from neutrophils in response to stimulation as well as decrease neutrophil phagocytic activity (Losche *et al*, 1996). Platelets are able to secrete transforming growth factor-β and the soluble from of P-selectin, both of which are able to inhibit adhesion of neutrophils to cultured endothelial cells (Gamble and Vadas, 1988; Gamble *et al*, 1990). Soluble P-selectin, together with PDGF, has the capacity to reduce superoxide anion generation by activated neutrophils. Bengtsson *et al* (1996) showed that resting platelets could limit the release of oxygen radicals from chemoattractant stimulated neutrophils. They were associated with an increase in the generation of neutrophil-derived adenosine, an inhibitor of respiratory burst, and peripheral accumulation of actin filaments forming a barrier for the extracellular release of reactive oxygen radicals. In addition, platelet production of nitric oxide (Radomski *et al*, 1990) and platelet-induction of its synthesis by endothelial cells (Vanhoutte and Miller, 1987) may inhibit neutrophil chemotaxis, aggregation and adhesion to endothelium (Kubes *et al*, 1991).

Neutrophil-dependent activation of platelets is related to production of superoxide anion, hydrogen peroxide, hypochlorous acid, elastase, cathepsin G and PAF (Bazzoni *et al*, 1991). Nitric oxide (Faint *et al*, 1991) and ADP-ases (Coade and Pearson, 1989) are particularly relevant when considering neutrophil-dependent inhibition of platelets.

CHAPTER 5

Proteases of the neutrophil membrane act as an alternative fibrinolytic pathway to that mediated by plasmin

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Introduction

The process of both blood coagulation and fibrinolysis needs to be finely controlled in order that the pathological states of uncontrolled bleeding or thromboembolism, with resultant ischaemia, do not ensue. This thesis is primarily concerned with fibrinolysis, a mechanism referring to the dissolution of fibrin clot and classically ascribed to the plasma zymogen plasminogen, which becomes activated to the proteolytic enzyme plasmin (Sawyer *et al*, 1960; Kaplan and Austen, 1972). Fibrinolytic activity, *in vivo*, is the result of a balance between plasminogen activator and inhibitor levels. However, in addition to the plasmin-mediated system of clot lysis, it has become apparent that the cellular constituents of the blood and in particular the neutrophil have an important role to play in contributing to fibrinolysis (Plow and Edgington, 1975). Elastase and cathepsin G, proteases present within the azurophilic granules of the cell and requiring relatively high activation states to be released, have both been described as possessing fibrinolytic properties (Plow, 1980). Mobilization of these granules is both a time and cell activation dependent process and fibrin would be more accessible as a substrate to proteases situated on the neutrophil membrane. Recently, a high molecular weight phorbol ester-upregulatable neutral protease, located at sites of attachment of the membrane with the cytoskeleton, has been demonstrated to possess fibrinogenolytic activity which is distinct from that of plasmin, neutrophil lysosomal enzymes and pure neutrophil elastase (Shephard *et al*, 1992; Kelly *et al*, 1994). Simultaneous early cleavage of all three constituent chains of fibrinogen to produce unclottable fibrinogen products, which do not exhibit anticoagulant activity, appears to be a characteristic of the fibrinogenolytic activity of this membrane-associated protease (Kelly *et al*, 1994).

Of further interest is that it has been found that leukocytes accumulate within fibrin clots due to specific cellular adhesive processes and not merely due to mechanical trapping (Palabrica *et al*, 1992). In the case of platelet rich clots accumulation is due in part to the specific interaction between P-selectin on activated platelets and its counterreceptor expressed on leukocytes. In the close interaction of fibrin(ogen) and neutrophils the transmembrane glycoproteins of the integrin

supergene family feature prominently. The C-terminal region of the γ chain of fibrinogen has been shown to bind to CD11b/CD18 (Wright *et al*, 1988). Altieri *et al* (1993) showed a calcium dependent binding of fibrinogen, involving the recognition motif $glycine_{190}$-$valine_{202}$ of the γ chain, to CD11b/CD18. PMA-stimulated neutrophil binding to the I domain of CD11b/CD18 occurs via a small subpopulation of approximately 10% of cells (Diamond and Springer, 1993; Diamond *et al*, 1993) while the interaction of TNF-stimulated neutrophils with immobilised fibrinogen is via the GPR sequence on the N-terminal domain of the Aα chain with CD11c/CD18 (Loike *et al*, 1991). This same integrin receptor appears to be important in the formation of a fibrinogen bridge between neutrophils and the platelet GPIIb-IIIa receptor (Ruf and Patscheke, 1995). In addition, the fibrinogen RDG and AKQAGDV sequences, in the α and γ chains respectively, as well as a novel KGAGDV sequence act as ligands for the LRI (Gresham *et al*, 1989; Gresham *et al*, 1992). To date no studies have investigated the interaction of the leukocyte specific β_2 integrins with fibrin and concomitant degradation of the ligand.

The aim of this study was to investigate the possible role that proteases of the neutrophil membrane may play in contributing to fibrinolysis and compare this to fibrinolysis by plasmin and the lysosomal enzyme component of the cell. Fibrinolysis by neutrophils and plasmin was quantified as the amount of soluble ^{125}I-labelled fibrin (cpm) released from the ^{125}I-labelled fibrin clot, expressed as a percentage of total ^{125}I-labelled fibrin (cpm) associated with the intact clot. The size of the products produced by the various enzyme sources was assessed by SDS-PAGE. Similarly, the fibrin degradation occurring without the addition of exogenous proteases was quantified and the stability of the clot over time was assessed by SDS-PAGE. Scanning and transmission electron microscopy was employed to monitor fibrin matrix alterations and morphological changes of the neutrophil. A variety of antibodies directed against the β_2 integrins were used to study their effect on neutrophil-mediated fibrin degradation under conditions of PMA-stimulation at concentrations not associated with azurophil granule release. In addition the possible signal transduction pathways involved in this process were also investigated using specific kinase inhibitors. The methodology used to obtain the results discussed in this chapter and chapters 6 and 7 may be found in appendix 1.

Results

Neutrophil fibrinolysis

In the absence of proteases (no lysosomal enzyme, intact cells or conditioned medium preparation) approximately 1% of the total radioactivity in the clot was released into the medium at 90 mins, indicating minimal spontaneous clot dissolution during the period of experimentation. SDS-PAGE (5-20%, reduced) followed by autoradiography shows the fibrin clot to contain both α and γ-γ crosslinking (Fig 1).

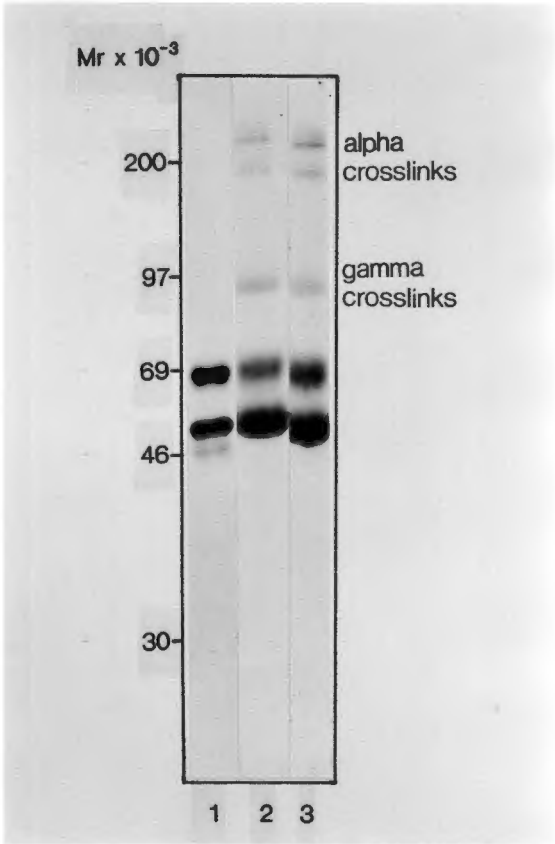
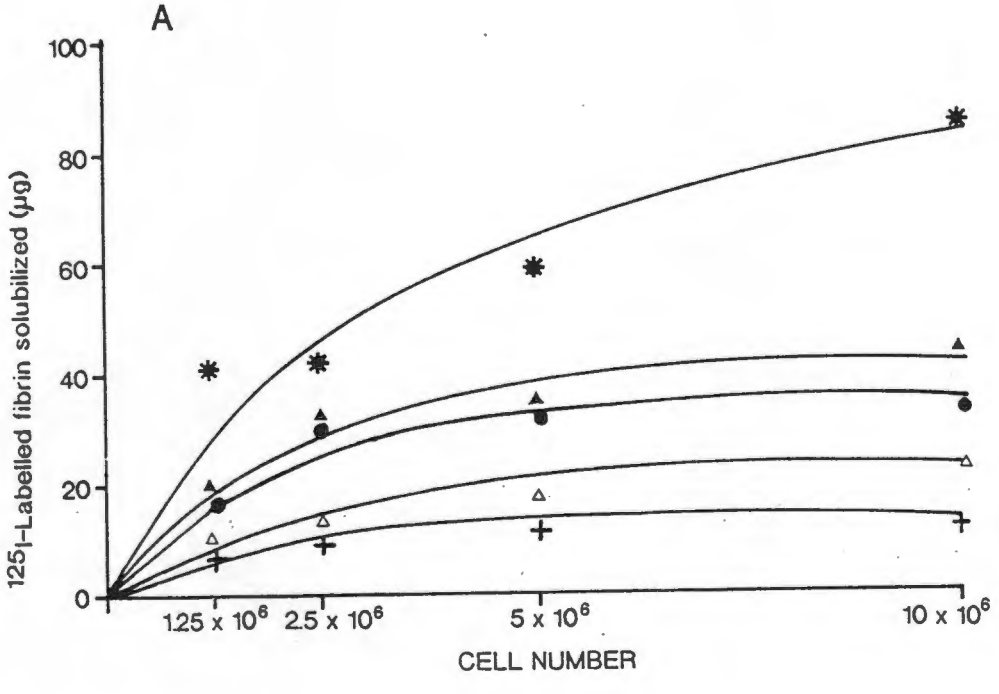
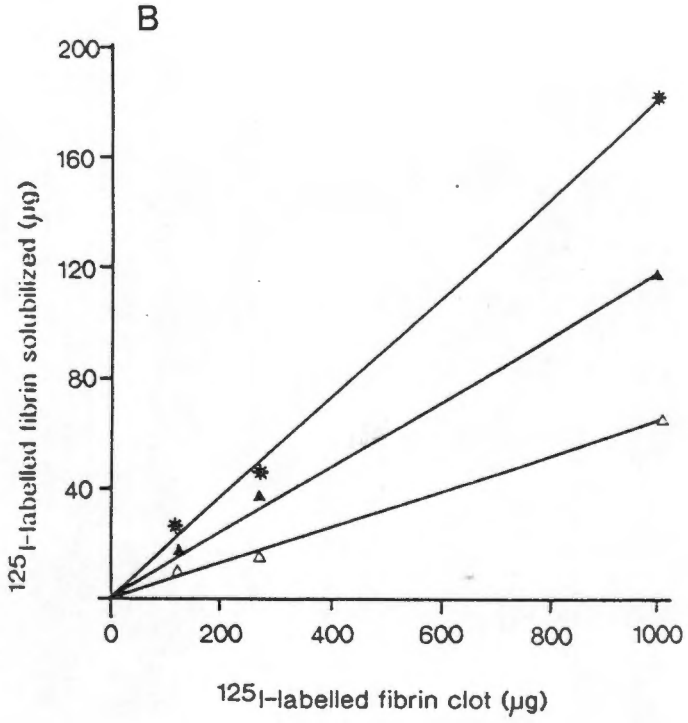


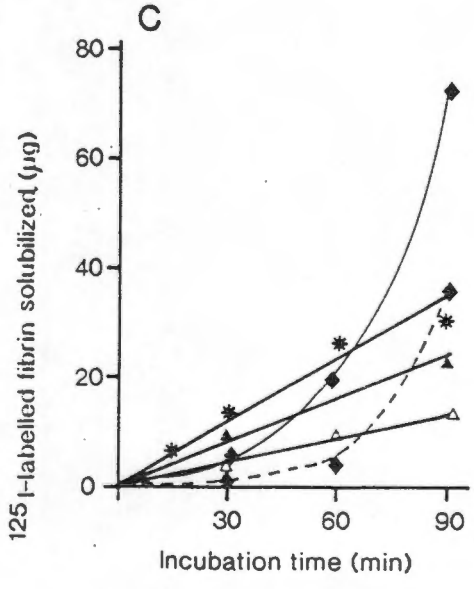
Figure 1. SDS-PAGE analysis (5-20% reduced) of fibrin clot formed by ^{125}I-labelled fibrinogen at a final concentration of 2 mg/ml HBSS with addition of bovine thrombin at 0.4 units/ml final concentration. Fibrinogen standard showing α (66 kDa), β (52 kDa) and γ (46.5 kDa) chains (track 1); fibrin clot 1 hr after formation showing both α-crosslinked polymer formation and γ-γ crosslinking (track 2), fibrin clot 24 hr after formation (track 3). Molecular weight markers as indicated.

The ability of non-stimulated neutrophils and neutrophils stimulated with a low dose of PMA, that does not release lysosomal enzymes from these cells (Shephard *et al*, 1989), to solubilize fibrin was evaluated. Solubilization of ^{125}I-labelled fibrin for any given concentration of cells was faster when the cells were stimulated with PMA (Fig 2A). The contribution of proteases released from neutrophils during incubation with ^{125}I-labelled fibrin to this solubilization was investigated by incubating ^{125}I-labelled fibrin with conditioned medium prepared from non-stimulated and PMA-stimulated neutrophils. Although PMA-stimulated neutrophil conditioned medium released more radioactivity than non-stimulated neutrophil conditioned medium, ^{125}I-fibrin solubilization in the presence of cells was 3 times and 2 times greater than that by the conditioned medium from non-stimulated and PMA-stimulated cells respectively (Fig 2A). When the solubilization of ^{125}I-labelled fibrin by intact neutrophils was compared to that by lysosomal enzymes, prepared from an equivalent number of cells, the latter enzyme source always released more radioactivity. The rate of ^{125}I-labelled fibrin solubilization by lysosomal enzymes from $10x10^6$ neutrophils was 2 fold that of $10x10^6$ PMA-stimulated cells (Fig 2A). For each of these neutrophil enzyme sources the release of radioactivity (37°C, 60 min) from clotted ^{125}I-labelled fibrinogen plateaued with high concentrations of each of these enzyme sources (Fig 2A) and was linear with increasing weights of ^{125}I-labelled fibrin (Fig 2B). The release of radioactivity from ^{125}I-labelled fibrin by each neutrophil source was detectable within 2 min and linear up to 90 min (Fig 2C). Solubilization of a ^{125}I-labelled fibrin clot (250 μg) by pure human neutrophil elastase (250 ng) was linear up to 45 min and reached completion in three hours. In contrast to the kinetics of clot solubilization by neutrophils, the kinetics of ^{125}I-labelled fibrin solubilization by plasmin was not linear. At time points prior to 60 min the rate of ^{125}I-labelled fibrin solubilization by plasmin was slower than that by the neutrophil enzyme sources but showed an exponential rise with time (Fig 2C). The extent of ^{125}I-labelled fibrin solubilization at 90 min by plasmin, generated from plasminogen at 5 μg/ml, equalled that by neutrophil lysosomal enzymes at 90 min (Fig 2C). The variation in clot solubilization for 10 different experiments and 10 different batches of ^{125}I-labelled fibrinogen varied by less than 10%.

Figure 2. Solubilization of a preformed ^{125}I-labelled fibrin clot. Each data point is the mean of triplicated determinations which did not vary by more than 2% and the illustrated results are one of ten replicate experiments. (A) With respect to enzyme levels at 60 min. Reactions (500 µl) contained the indicated number of neutrophils or neutrophil equivalents and 250 µg preformed ^{125}I-labelled fibrin. Lysosomal enzyme (✱), intact PMA-stimulated neutrophils (▲), intact non-stimulated neutrophils (●), conditioned medium from PMA-stimulated neutrophils (Δ), conditioned medium from non-stimulated neutrophils (+). (B) With respect to clot size at 60 min. Reactions (500 µl) contained $2.5x10^6$ neutrophils or neutrophil equivalents and the indicated weight of preformed ^{125}I-labelled fibrin. Lysosomal enzymes (✱), intact PMA-stimulated neutrophils (▲), conditioned medium from PMA-stimulated neutrophils (Δ). (C) With respect to time. Reactions (500 µl) contained 125 µg preformed ^{125}I-labelled fibrin and either $2.5x10^6$ neutrophils or neutrophil equivalents or plasmin generated by activation of 5 µg or 15 µg plasminogen/ml with 0.4 IU urokinase/ml. Lysosomal enzymes (✱), intact PMA-stimulated neutrophils (▲), conditioned medium from PMA-stimulated neutrophils (Δ), plasmin (from 5 µg plasminogen) (– –◆– –), plasmin (from 15 µg plasminogen) (—◆—).




B
200
160
120
80
40
0
200
400
600
800
1000
125I-labelled fibrin solubilized (µg)
125I-labelled fibrin clot (µg)


C
80
60
40
20
0
30
60
90
125I-labelled fibrin solubilized (µg)
Incubation time (min)

Fibrinolysis by neutrophils incorporated into the clot

The extent of ^{125}I-labelled fibrin solubilization by neutrophils, neutrophil conditioned medium and lysosomal enzymes incorporated into the clot at 60 min was compared with that when these enzyme sources were added to ^{125}I-labelled fibrin (Fig 3). Incorporation of each enzyme source (neutrophil conditioned medium from non-stimulated or PMA-stimulated neutrophils, non-stimulated or PMA-stimulated neutrophils, or lysosomal enzymes) into the clot resulted in the solubilization being 3.8, 4.4, 3.0, 3.4 and 3.4 times greater at 60 min than when these respective enzyme sources were added to the clot (Fig 3). The release of radioactivity from ^{125}I-labelled fibrin by lysosomal enzymes incorporated into the clot was 2.0 or 1.7 times greater respectively than that by an equivalent number of non-stimulated or PMA-stimulated neutrophils that had been incorporated into the clot (Fig 3). Complete solubilization of the clot was achieved within 60 min when PMA-stimulated cells were incorporated into the clot at a concentration of $10x10^6$ cells/mg ^{125}I-labelled fibrin (data not shown). The variation in clot solubilization for 10 different experiments and 8 batches of ^{125}I-labelled fibrinogen, when these neutrophil enzyme sources were incorporated into the clot, was less than 10%.

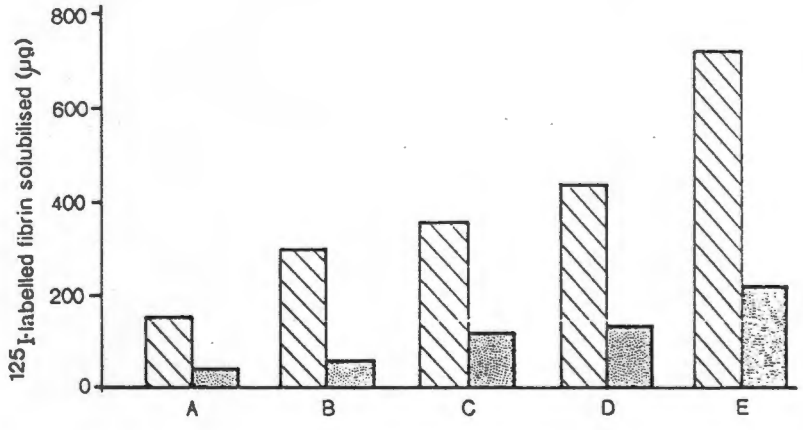


Figure 3. Comparison of clot solubilization by neutrophils added to a preformed 1 mg ^{125}I-labelled fibrin clot (▒) and neutrophils incorporated into a 1 mg ^{125}I-labelled matrix (\\ ). Reactions contained either $2.5x10^6$ neutrophils. conditioned medium from $2.5x10^6$ neutrophils or lysosomal enzymes from $2.5x10^6$ neutrophils in a final volume of 500 µl. A: conditioned medium from non-stimulated neutrophils, B: conditioned medium from PMA-stimulated neutrophils. C: intact non-stimulated neutrophils. D: intact PMA-stimulated neutrophils. E: neutrophil lysosomal enzymes. Each data point is the mean of triplicate determinations which did not vary by more than 2% and the illustrated results are one of ten replicate experiments.

The effect of neutrophils on plasmin solubilization of ^{125}I-labelled fibrin

PMA-stimulated neutrophils and PMA-stimulated neutrophil conditioned medium enhanced the solubilization of ^{125}I-labelled fibrin by plasminogen activated by exogenous urokinase (Fig 4). This effect was observed when neutrophils were added to the clot with plasmin at zero time and with the addition of neutrophils after plasminogenolysis had progressed for 30 min (Fig 4). When plasminogenolysis of a preformed ^{125}I-labelled fibrin clot was inhibited with aprotinin (final concentration 20 units/ml) and ε-amino-n-caproic acid (EACA, 25 mM) at 30 min, solubilization of the remaining clot by the addition of PMA-stimulated neutrophils was 2.7 times greater at 90 minutes than that by neutrophils alone (Fig 4). Three separate experiments gave results within 10% of those demonstrated in Fig 4.

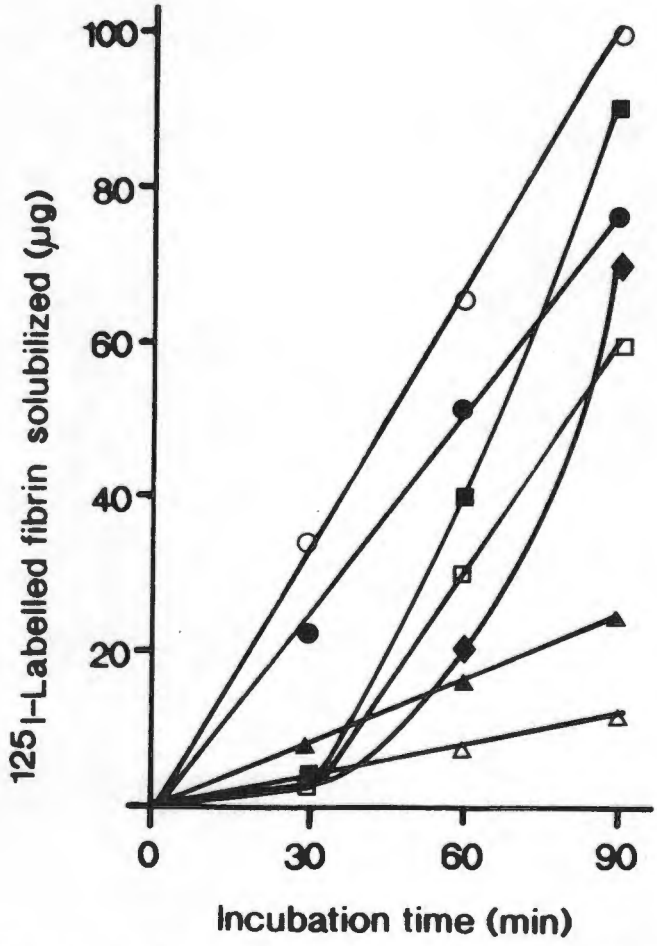


Figure 4. Influence of PMA-stimulated neutrophils ($2.5x10^6$) or PMA-stimulated neutrophil conditioned medium (from $2.5x10^6$ neutrophils) on plasmin solubilization of a 125 µg preformed ^{125}I-labelled fibrin clot in a final volume of 500 µl. Plasmin was generated by the addition of urokinase (50 IU/ml final concentration) to 15 µg plasminogen/ml. PMA-stimulated neutrophils (▲), conditioned medium from PMA-stimulated neutrophils (Δ), plasmin (◆), PMA-stimulated neutrophils (○) and PMA-stimulated neutrophil conditioned medium (●) together with plasmin at '0' min, PMA-stimulated neutrophils with plasmin added at 30 min (■), PMA-stimulated neutrophils (□) added to the clot solubilized with plasmin for 30 min then inhibited with EACA (25 mM) and aprotinin (20 U/ml). Each point is the mean of triplicates that did not differ by more than 2% and the illustrated results are one of three replicate experiments whose results were within 10% of those demonstrated.

Nature of soluble ^{125}I-labelled fibrin products

The products produced on full solubilization of a preformed ^{125}I-labelled fibrin clot by PMA-stimulated neutrophils, PMA-stimulated neutrophil conditioned medium, neutrophil lysosomal enzymes, purified human neutrophil elastase and plasmin are shown in Fig 5. The apparent molecular mass of the products formed by PMA-stimulated neutrophils and the conditioned medium from these cells were similar, but different from the products produced by lysosomal enzymes and pure human neutrophil elastase (Fig 5). Plasmin generated soluble ^{125}I-labelled fibrin products of different molecular mass to those produced by neutrophil enzymes (Fig 5).

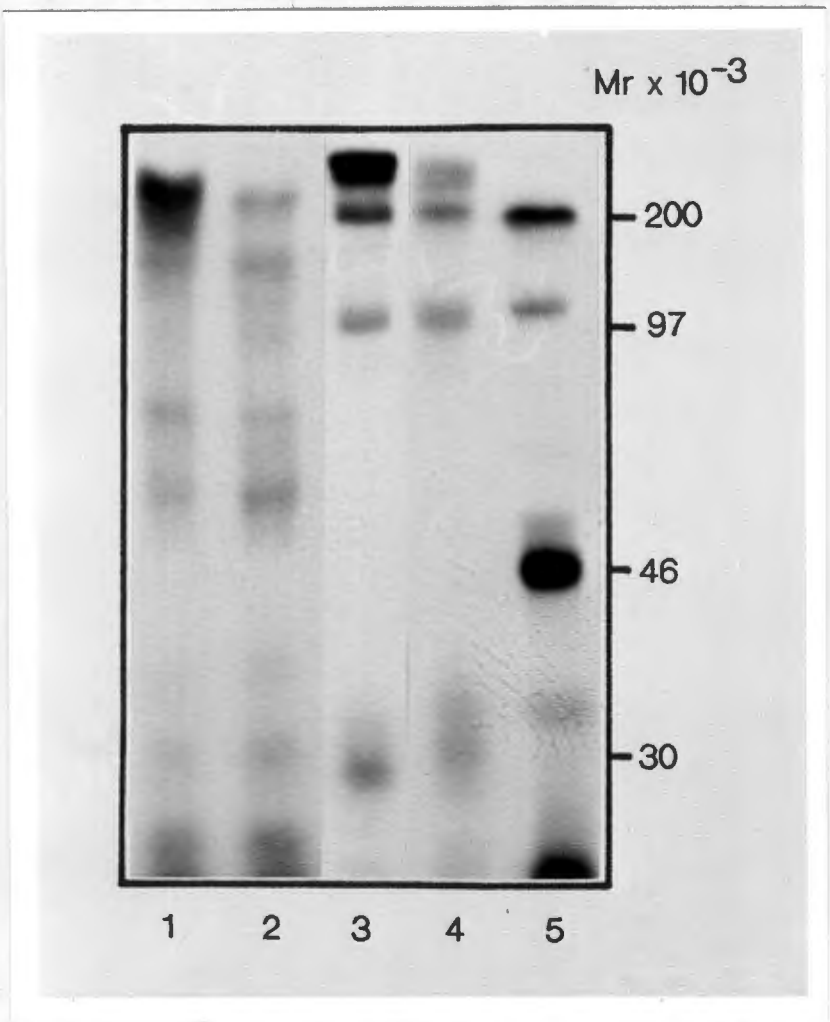


Figure 5. SDS-PAGE analysis (10% non-reduced) of the ^{125}I-labelled protein in solution on full solubilization of a 250 μg ^{125}I-labelled fibrin clot in a final volume of 500 μl by PMA-stimulated neutrophils ($2.5x10^6$, track one), neutrophil conditioned medium from PMA-stimulated neutrophils (from $2.5x10^6$ neutrophils, track two), lysosomal enzymes (from $2.5x10^6$ neutrophils, track three) incorporated into the clot, pure human neutrophil elastase (0.25 μg, track four) or plasmin (generated by the addition of 50 IU urokinase to 15 μg plasminogen/ml, track five) added to a preformed clot.

Effect of inhibitors on the solubilization of ^{125}I-labelled fibrin

No significant inhibition of solubilization of a preformed clot by PMA-stimulated neutrophils was achieved with inhibitors of the plasmin system - EACA (25 mM) and aprotinin (20 units/ml), EDTA (5 mM), a thiol protease inhibitor - E64 (20 mM), the cathepsin G inhibitors Suc-$(Ala)_2$-Pro-PheCH_2Cl (1 mM) and Z-Gly-Leu-PheCH_2Cl (0,1 mM) or the human neutrophil elastase inhibitors Suc-$(Ala)_2$-ValCH_2Cl (1 mM) and Suc-$(Ala)_3CH_2Cl$ (1 mM) (Table 1). 4-(2-aminoethyl)-benzene sulfonyl fluoride (AEBSF, Pefabloc® SC - Boehringer Mannheim, Germany, 1 mM), a water soluble serine protease inhibitor which is non-toxic to cells, inhibited fibrin degradation by 95%, while MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM), an inhibitor of proteases such as elastase with specificities related to alanine or valine bonds, inhibited degradation by 35% (Table 1). Using a neutrophil lysosomal enzyme preparation to degrade preformed fibrin clots showed a very different inhibition profile, with MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM) producing 80% inhibition, Z-Gly-Leu-Phe-CH_2Cl (0.1 mM) 44% inhibition and AEBSF (1 mM) 90% inhibition (Table 1). MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM) inhibited the solubilization of a preformed clot (250 μg) by pure human neutrophil elastase (250 ng) by 97% (Table 1). When neutrophils were incorporated into a 1 mg clot 440 μg ^{125}I-labelled fibrin was solubilized in 60 min. This solubilization was inhibited by 30% by MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM) but not inhibited by the cathepsin G inhibitor, Z-Gly-Leu-PheCH_2Cl (0.1 mM). These inhibitors did not inhibit clot formation by thrombin.

Identification of proteases in a neutrophil membrane preparation capable of degrading fibrin

The fibrinolytic activity associated with the neutrophil membrane was only recovered from regions of the SDS-PAGE that contained proteins which migrated to apparent molecular weights of 501 kD, 398 kD, 316 kD, 245 kD and 209 kD. These slices released 230 μg, 205 μg, 225 μg, 160 μg and 160 μg of ^{125}I-labelled fibrin from a 250 μg fibrin clot respectively at 24 hours.

Table 1. Influence of inhibitors on ^{125}I-labelled fibrin degradation by neutrophils.

Inhibitor	^{125}I-labelled fibrin solubilized (μg)
PMA-stimulated neutrophils	
None	38
EACA (25 mM)	38
Aprotinin (20 units/ml)	37
EDTA (5 mM)	38
E64 (20 mM)	38
Suc-$(Ala)_2$-Pro-PheCH_2Cl (1 mM)	38
Z-Gly-Leu-PheCH_2Cl (0.1 mM)	36
Suc $(Ala)_2$-Val-CH_2Cl (1 mM)	37
Suc-$(Ala)_3CH_2Cl$ (1 mM)	37
AEBSF (1 mM)	2
MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM)	24
Neutrophil lysosomal enzymes	
None	50
Z-Gly-Leu-PheCH_2Cl (0.1 mM)	28
AEBSF (1 mM)	5
MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM)	10
Pure human neutrophil elastase	
None	70
AEBSF (1 mM)	2
MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM)	2

A preformed ^{125}I-labelled fibrin clot (250 μg) was incubated (60 min) with PMA-stimulated neutrophils ($2.5x10^6$), neutrophil lysosomal enzymes (from $2.5x10^6$ neutrophils) and pure human neutrophil elastase (250 ng) in a final volume of 500 μl. The inhibitors were incubated with the enzyme source for 15 min prior to the addition of the clot. Solubilized ^{125}I-labelled fibrin is expressed as μg ^{125}I-labelled fibrin/500 μl and is the mean of triplicate determinations which did not vary by more than 2%. The illustrated results are one of four replicate experiments.

Electron microscopy of fibrinolysis by neutrophils incorporated within the fibrin matrix

Scanning Electron Microscopy. The scanning electron micrograph of the fibrin network in the absence of incorporated neutrophils revealed fibres organized into a tight matrix (Fig 6a) and incorporation of the neutrophils into the fibrin matrix occurred with an even distribution (Fig 6b). When non-stimulated neutrophils were incorporated into the clot a time dependent opening of the network in the vicinity of the cells was observed (Fig 6c). After a 10 min incubation period the neutrophils were still emeshed within the fibrin matrix (Fig 6d) but by 60 min many of the neutrophils were found to be divest of fibrin and gaps within the matrix were visible (Fig 6e). No formation of focal adhesions of the neutrophils were observed and the cells appeared to remain round with no spreading on the fibrin matrix, although pseudopodia formation was evident (Fig 6f). Electron microscopy analysis of fibrinolysis by PMA-stimulated neutrophils incorporated into the fibrin matrix indicated the process to be similar to that by non-stimulated neutrophils. From the micrographs it appeared that the time required for gaps to form in the fibrin matrix and the neutrophils to become divest of fibrin was far faster when PMA-stimulated neutrophils were incorporated into the matrix (Fig 6g) than when non-stimulated neutrophils were incorporated into the matrix (Fig 6e).

Transmission Electron Microscopy. Non-stimulated neutrophils incorporated into the fibrin matrix for 60 min, at which time extensive fibrinolysis has occurred, contained a high concentration of dense granules (Fig 7a). No evidence of release of these granules into the extracellular medium was seen. Examination of the cells at high power revealed extensive ruffling of the membrane and pseudopodia formation (Fig 7b). Analysis of PMA-stimulated neutrophils that had been incorporated into the clot for a 60 minute period revealed similar membrane activation without granule release (Fig 7c).

Figure 6. (A) Appearance on scanning electron microscopy of fibrin network with no neutrophils incorporated within the matrix. (Magnification 2700x). (B) Incorporation of neutrophils into fibrin matrix showing an even distribution of cells using scanning electron microscopy (Magnification 1125x). (C) Incorporation of neutrophils into fibrin matrix at 10 min showing opening up of the fibrin network in the vicinity of the cells using scanning electron microscopy (Magnification 1800x). (D) Appearance on scanning electron microscopy of neutrophils within the fibrin network at 10 min incorporation time (Magnification 4500x). (E) After 60 min it is evident that the cell is divest of fibrin and gaps within the matrix are present (Magnification 4500). (F) Scanning electron microscopy shows that the cells are rounded with no spreading of the neutrophil on the fibrin substrate, although pseudopodia formation is present (Magnification 3375x). (G) Scanning electron micrograph of a PMA-stimulated neutrophil emeshed within fibrin at 10 min incorporation time (Magnification 3150x).

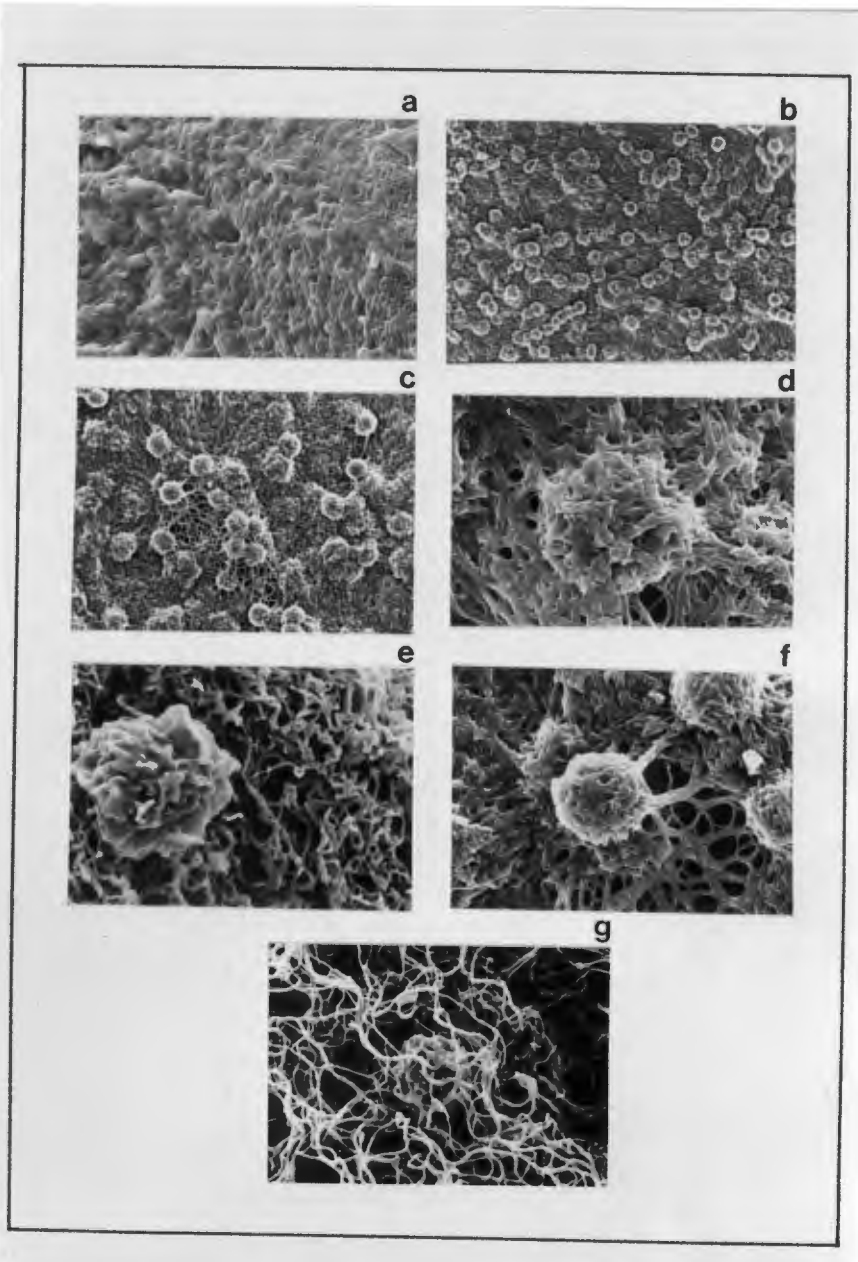
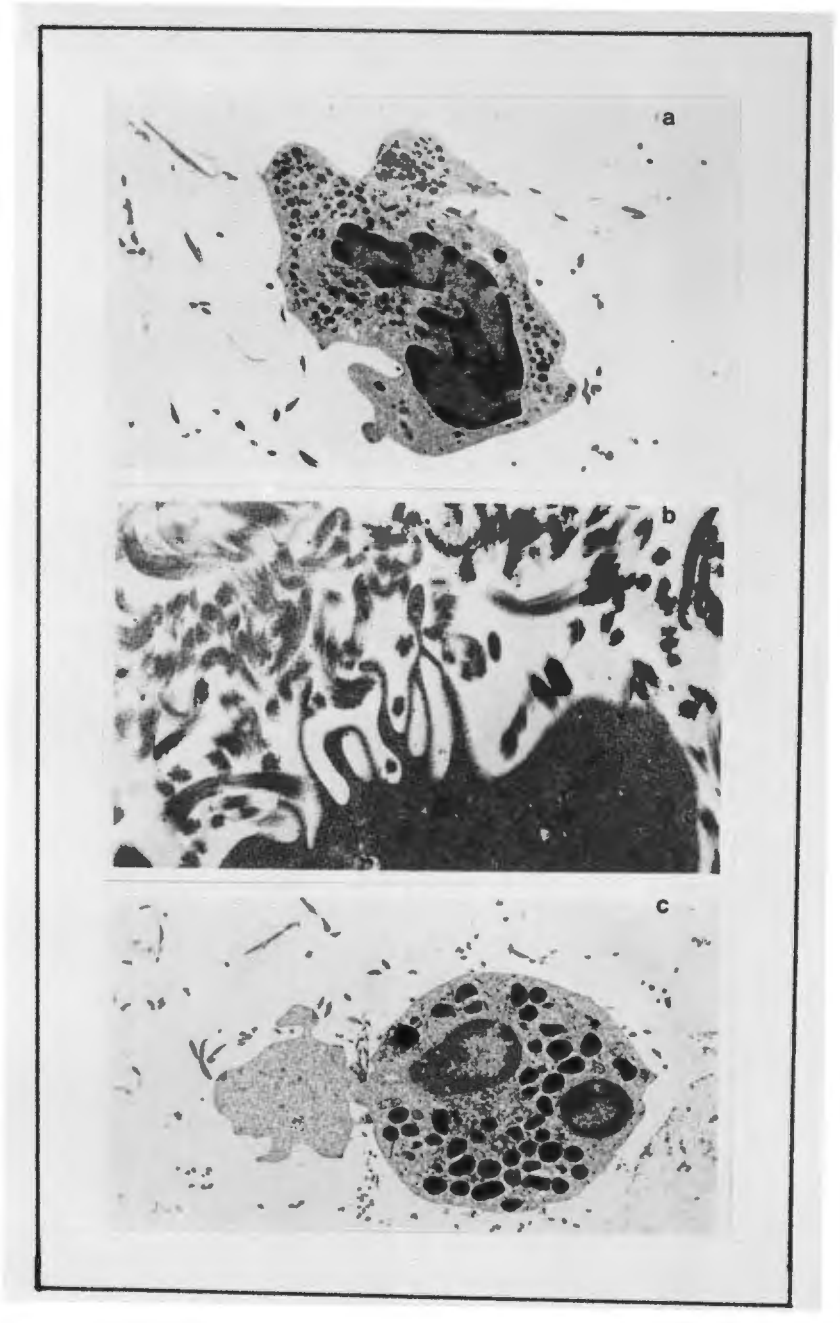


Figure 7. (A) Transmission electron microscopy shows a neutrophil at 60 min incorporation time with a high concentration of dense granules within the cytoplasm and no evidence of extracellular release. (B) Transmission electron microscopy of the neutrophil membrane in the vicinity of the fibrin substrate shows ruffling and pseudopodia formation. (C) Transmission electron micrograph of a PMA-stimulated neutrophil after 60 min incorporation into a fibrin clot, when extensive degradation has occurred. There is a high concentration of dense granules within the cytoplasm and similar membrane activation to that seen in neutrophils exposed to fibrin but not to PMA.



Mechanism of neutrophil-mediated fibrin degradation

(1) Fibrin releases proteolytic activity from neutrophils

Solubilization of clots by PMA-stimulated neutrophils was faster than that by conditioned medium from PMA-stimulated cells. This might be due to the ability of fibrin to release proteolytic activity from the neutrophil. To investigate this, PMA-stimulated neutrophils ($2.5x10^6$ and $5x10^6$ cells) were incorporated into a 1 mg fibrin clot and degradation allowed to proceed for 90 min. Experiments were performed with both ^{125}I-labelled fibrin and unlabelled fibrin. After 90 min the extent of fibrin degradation in the reaction containing ^{125}I-labelled fibrin was assessed as described above. The ability of fibrin to release proteolytic activity from the neutrophil during a period of 90 min was assessed by incorporating the cell-free and fibrin-free supernatant obtained from the reaction containing unlabelled fibrin into the preparation of a 1 mg ^{125}I-labelled fibrin clot. The subsequent fibrinolysis occurring over 60 min was calculated and compared to that by conditioned medium prepared for 90 min from PMA-stimulated neutrophils not previously exposed to fibrin substrate. PMA stimulation was done at a concentration of 10 ng/ml, a concentration not associated with azurophil granule release (Shephard *et al*, 1989). The results are illustrated in Table 2.

Table 2. Fibrin solubilization by intact neutrophils and conditioned medium incorporated into fibrin matrix (1 mg).

$2.5x10^6$ neutrophils/1 mg fibrin clot	**$5x10^6$ neutrophils/1 mg fibrin clot**
^{125}I-labelled fibrin solubilized by PMA-stimulated neutrophils at 90 min (μg)	
650	910
^{125}I-labelled fibrin solubilized at 60 min by conditioned medium from PMA-stimulated neutrophils exposed to fibrin for 90 min (μg)	
396	432
^{125}I-labelled fibrin solubilized at 60 min by conditioned medium from PMA-stimulated neutrophils (μg)	
260	308

It is clear that conditioned medium from PMA-stimulated neutrophils exposed to fibrin is more efficient at subsequent fibrinolysis than conditioned medium from neutrophils stimulated with PMA alone.

(2) Integrin receptors and cellular kinases are involved in neutrophil-mediated fibrin degradation

(a) Integrin receptors

The effect of antibodies directed against various leukocyte receptors on neutrophil-mediated fibrin degradation by cells stimulated with PMA, at a concentration that does not result in azurophil granule release, is shown in Table 3. The concentration of monoclonal antibodies required to saturate these receptors on PMA-stimulated neutrophils was pre-determined by flow cytometry. The control antibody directed against the platelet receptor GPIIb-IIIa (CD41) and that directed against the neutrophil IgG Fc receptor (CD16), important in phagocytosis, produced no inhibition of fibrin degradation at 60 min. Pre-incubation of PMA-stimulated neutrophils with monoclonal antibodies directed against the α and β subunits of the leukocyte specific β_2 integrins before incorporation into a fibrin clot were able to inhibit degradation by varying amounts. Anti-CD11a, -CD11b, -CD11c and -CD18 produced 15%, 13%, 58% and 58% inhibition of degradation at 60 min respectively (Table 3).

Table 3. Influence of monoclonal antibodies directed against various neutrophil receptors on I^{125}-labelled fibrin degradation (60 mins) by PMA-stimulated neutrophils ($2.5x10^6$ neutrophils/1 mg fibrin clot). Monoclonal antibodies incubated (37°C, 15 min) with PMA-stimulated (10 ng/ml) neutrophils before addition of I^{125} -labelled fibrinogen and thrombin (0.4 units/ml) to effect clot formation. Degradation was monitored by release of radioactivity into supernatant at 60 min. In the absence of antibody 450 μg ^{125}I-labelled fibrin was released into the medium at 60 min. The results represent the mean of triplicate experiments that did not vary by more than 5%.

Monoclonal antibody	% Inhibition of degradation
none	0
anti-CD11a	15
anti-CD11b	13
anti-CD11c	58
anti-CD18	58
anti-CD16	0
anti-CD41	0

(b) Cellular kinases

Protein phosphorylation represents one of the most important molecular mechanisms by which extracellular signals produce their biological reponses in cells (Cohen, 1992). In view of this, specific kinase inhibitors were used to assess their role in the degradation process: ML-9, an inhibitor of the myosin light chain kinase; KN-62, an inhibitor of the calcium/calmodulin dependent kinase and bisindolylmaleimide, a specific protein kinase C inhibitor (Ito *et al*, 1989; Tokumitsu *et al*, 1990; Toullec *et al*, 1991). From the results presented (Table 4) it is clear that protein kinase C plays a fundamental role in the signal transduction pathway leading to fibrin degradation.

Table 4. Influence of various kinase inhibitors on fibrin degradation by non-stimulated and PMA-stimulated neutrophils at 60 min ($2.5x10^6$ neutrophils/1 mg fibrin clot). Kinase inhibitors incubated (37°C, 15 min) with non-stimulated and PMA-stimulated (10 ng/ml) neutrophils before the addition of thrombin (0.4 units/ml) and I^{125}-labelled fibrinogen. Degradation was monitored by the release of radioactivity into the supernatant. In the absence of inhibitors non-stimulated neutrophils released 120 μg of ^{125}I-labelled fibrin and stimulated cells released 450 μg of ^{125}I-labelled fibrin into the medium at 60 min. The results represent the mean of triplicate experiments that did not vary by more than 4%. The results of three experiments did not vary by more than 10%.

Inhibitor	% Inhibition (-PMA)	% Inhibition (+PMA)
None	0	0
ML-9 (20 μM)	0	14
Bisindolylmaleimide (5 μM)	10	34
KN-62 (10 μM)	3	17

Discussion

The incorporation of leukocytes into areas of clot formation *in vivo* is an active process mediated by P-selectin expressed on actived platelets (Palabrica *et al*, 1992) which binds to its ligand, PSGL-1, on monocytes and neutrophils (Larson *et al*, 1989). In addition, the presence of uPA-R on the neutrophil membrane localises uPA to the cell surface and may facilitate their infiltration into the clot (Herijgers *et al*, 1995). The presence of leukocytes with their substantial array of proteolytic enzymes possibly contributes to the recognized plasminogen independent fibrinolytic pathways (Plow and Edgington, 1975; Moroz and Gilmore, 1976; Moroz, 1984). Studies using leukocyte lysates as a source of enzymes have ascribed leukocyte fibrinolysis to neutrophil cathepsin G and elastase (Plow, 1980) and monocyte cathepsin D (Simon *et al*, 1994). A distinct difference in the overall molecular size of the degradation products from cross-linked fibrin produced by plasmin and leukocyte lysates has been reported (Francis and Marder, 1986). Leukocyte-derived elastase may contribute indirectly to accelerated fibrinolysis through the generation of a plasminogen-derived mini-plasminogen which can be activated by urokinase to yield mini-plasmin (Moroz, 1981). Mini-plasmin has been shown to have enhanced fibrinolytic activity compared to plasmin and to be relatively resistant to inhibition by the primary plasmin inhibitor α_2-antiplasmin (Moroz, 1981). In the absence of plasminogen, neutrophil elastase may be a major contributor to non-plasmin mediated fibrinolysis. Kolev *et al* (1996) looked at the relative contributions of plasmin, miniplasmin, neutrophil elastase and cathepsin G to the process of fibrin degradation. No quantitative difference in fibrin degradation was found between plasmin and mini-plasmin present on the surface of preformed clots, but elastase and cathepsin G were less efficient. In the case of fibrin crosslinking by factor XIIIa in the presence of α_2-antiplasmin, clot resistance to plasmin and mini-plasmin is increased while the action of leukocyte proteases is not affected. Under the same conditions both leukocyte proteases were also able to potentiate the action of plasmin, possibly by its conversion to mini-plasmin. Degradation of immobilized fibrinogen by intact neutrophils has been shown to occur through the release of elastase as a result of the formation of a protected compartment, which excludes plasma protease inhibitors, at the fibrinogen-cell interface (Weitz *et al*, 1987). This occurs due to binding of fibrinogen to neutrophils via the β_2 integrins (Loike *et al*, 1992). Following degranulation and release of elastase and cathepsin G into the extracellular enviroment these enzymes have been shown to rebind to the cell membrane in an active form that is relatively resistant to proteinase inhibitors (Bangalore and Travis, 1994; Owen *et al*, 1995a).

To date no study has addressed the role enzymes of membrane origin play in fibrinolysis when intact leukocytes lyse fibrin clots or become incorporated into a clot. In this study we show that during incubation of non-stimulated neutrophils and neutrophils stimulated with a dose of PMA that does not cause azurophilic granule exocytosis (Shephard *et al*, 1989), AEBSF sensitive fibrinolysis occurred. Neutrophil-mediated fibrinolysis produced products that were distinctly different from that of plasmin and were insensitive to plasmin inhibitors, EDTA, thiol protease inhibitors and specific cathepsin G inhibitors. Although partial inhibition (35%) of clot solubilization was observed with the specific elastase inhibitor MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl, it was insensitive to two other specific elastase inhibitors. Analysis of the apparent molecular masses of the solubilized products suggests neutrophil-mediated and neutrophil conditioned medium solubilization of ^{125}I-labelled fibrin occurs via similar enzymes and not by neutrophil lysosomal enzymes or elastase.

The results with protease inhibitors together with our finding that fibrinolysis in the presence of neutrophils was always greater than that by neutrophil conditioned medium and detectable within 2 min, suggest that clot solubilization in the presence of neutrophils is predominantly a neutrophil-mediated process occurring by a membrane-associated protease. Our finding that a neutrophil membrane preparation contains fibrin solubilizing activity further supports this conclusion. In addition fibrin appears to cause a release of proteolytic activity from the neutrophil which could assist degradation in the extracellular medium. This neutrophil membrane protease appears to be the same as a previously reported membrane-associated protease, partially resistant to MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl inhibition and with proteolytic activity of an apparent molecular weight greater than 200 kD, that degrades both C-reactive protein and fibrinogen during incubation with neutrophils (Shephard *et al*, 1992; Kelly *et al*, 1994). This reported protease was found to have an apparent molecular weight of 600 kDa on gel filtration and to dissociate into distinct subunits that migrated to apparent molecular weights greater than 200 kDa on SDS-PAGE. The location of this enzyme was proposed to be at sites of interaction of the membrane with the cytoskeleton, since the enzyme was found associated with a neutrophil cytoskeleton preparation (Kelly *et al*, 1994).

Electron microscopy analysis of clot lysis showed the fibrinolytic process to be localized to the vicinity of the cells without the involvement of azurophilic granule release. The number of available binding sites for neutrophils on an intact clot appears to be limited. However incorporation of neutrophils into the clot, which is analogous to an *in vivo* process during thrombus formation, increased the rate of fibrinolysis by cell-surface proteases and complete solubilization of the clot was achieved within 60 min with high neutrophil concentrations. The observed degradation of fibrin by

the membrane-associated proteases of the neutrophil, as measured by release of radioactivity from the clot, was marginally sensitive to upregulation by stimulating the cells with PMA. However, electron microscopy of clot solubilization suggests that the organization of the fibrin matrix is lost more readily when neutrophils are stimulated with PMA. This does not appear to be due to the release of lysosomal enzymes as no evidence was obtained either by the use of lysosomal enzyme inhibitors, analysis of the nature of the final fibrin products on complete solubilization of the clot or by transmission electron microscopy analysis of the solubilizing clot.

Neutrophils appear to enhance the action of plasmin on clot lysis, a system that would be expected to occur *in vivo*. The resistance of platelet rich clots to lysis by plasminogen activators has been ascribed to a variety of mechanisms including the production of PAI-1 by platelets (Booth *et al*, 1992), release of α_2-plasmin inhibitor by platelet α-granules (Sakata and Aoki, 1980), release of factor XIII from platelets leading to increased cross-linking of the fibrin network as well as cross-linking of α_2-antiplasmin to the fibrin clot (Reed *et al*, 1991; Francis and Marder, 1987). Although controversial it appears that the release of PAI-1 represents the most important mechanism. We show in this study that initial plasmin attack of the fibrin clot allows neutrophil sensitive sites to be exposed and subsequently a more efficient solubilization of the clot by neutrophils occurs.

The β_2 class of integrins and the "β_3-like" LRI have been shown to be important as receptors on the neutrophil for interaction with fibrinogen (Gresham *et al*, 1992; Wright *et al*, 1988; Altieri *et al*, 1993; Loike *et al*, 1991). The monoclonal antibody studies undertaken in this thesis did not determine binding sites on the fibrin matrix for neutrophils. However, the binding of monoclonal antibodies directed against either CD11c or CD18 was able to significantly inhibit subsequent neutrophil-mediated fibrinolysis, with the monoclonal antibodies directed against the CD11a and CD11b having a much smaller inhibitory effect on subsequent fibrinolysis. The implication is that the membrane protease mediating fibrinolysis must be situated close to or at the site of the CD11c/CD18 receptor as occupation of this receptor by antibody inhibits the degradation process, possibly interfering with enzyme/substrate interaction. The linkage of integrin receptors to the cytoskeleton of the cell has been widely reported (Pavalko and Otey, 1994; Wang *et al*, 1993). The protease important in the fibrinolytic process as stated above is more than likely localised at the site of attachment of the cytoskeleton with the cell membrane (Shephard *et al*, 1992). It is tenable therefore that CD11c/CD18 is the receptor for crosslinked fibrin clot and occupation by substrate initiates transduction pathways resulting in cytoskeletal reorganization

within the cell, upregulation of enzyme activity and release of enzyme activity into the extracellular medium.

The upregulation of activity of the neutrophil membrane-associated protease that occurs following the interaction of fibrin with neutrophils, appears to be due to the activation of cellular kinases and in particular PKC, as use of bisindolylmaleimide results in significant inhibition of fibrinolysis. Stimulation with PMA enhances the activity of the enzyme possibly through further activation of PKC. This serine/threonine kinase was first characterized on the basis of its activation *in vitro* by calcium, phospholipid and diacylglycerol (Nishizuka, 1992) and has subsequently been found to consist of a number of isotypes that have separate and unique functions within the cell (Dekker and Parker, 1994). PKC is thought not to phosphorylate integrin receptors directly but acts on cytoskeletal proteins thereby affecting the interaction of integrins with these intracellular proteins (Danilov and Juliano, 1989). It has been shown that PKC is necessary for spreading and adhesion of human monocytes on fibrinogen (Kreuzer *et al*, 1996). Use of specific inhibitors indicates that the activity of the myosin light chain kinase, important in microfilament assembly, and the calcium/calmodulin-dependent kinases are far less important in the modulation of fibrinolytic activity of the membrane proteases of the neutrophil.

From the results presented it may be concluded that neutrophil membrane proteolytic systems, which require relatively low activation states and act together with plasmin, could play an important role in early clot dissolution. The fibrinolytic processes described are due in part to the activation of PKC following binding of substrate at a site in close proximity to CD11c/CD18. In contrast the fibrinolytic activity of neutrophil lysosomal enzymes possibly only plays a role in the late inflammatory response following their mobilization, which is a time and cell activation dependent process. In the event of clot stabilization as a result of inhibition of plasminogen activators by PAI-1, complete fibrinolysis appears to be achieved through the further action of neutrophils. Thus neutrophil membrane proteolytic activity could play a major role in controlling fibrin deposition and thrombus dissolution.

CHAPTER 6

Modification of platelet function by fibrin degradation products generated by neutrophil membrane proteolytic activity on fibrin

CHAPTER 6

Modification of platelet function by fibrin degradation products generated by neutrophil membrane proteolytic activity on fibrin

Introduction

In vivo, a thrombus is made up of fibrin and a variety of cellular constituents including neutrophils, monocytes and platelets. These different components are associated with each other via a myriad of complex interactions which in turn influences clot structure, tension, which is related to the clot retraction process, and lysis.

Resistance to plasmic lysis is a feature of platelet-rich thrombi (Falk, 1992). The reasons for this are manyfold. Platelet release of factor XIII causes crosslinking of fibrin monomers which are more difficult to lyse than non-crosslinked monomers (Reed *et al*, 1991; Francis and Marder, 1987). Clot tension and retraction is also dependent on the interaction of the platelet receptor GPIIb-IIIa with polymerizing fibrinogen. A consequence of this binding is the contraction of the microfilaments of the platelet cytoskeleton and alignment and orientation of fibrin strands which causes the clot to retract, the extent of which determines the tension of the fibres of the clot (Kunitada *et al*, 1992). During this process the surface of the platelet becomes coated with compact fibrin and thus local areas of high density fibrin are created within the clot (Braaten *et al*, 1994). Retardation of clot lysis at the platelet surface appears to be the consequence of the exclusion of plasminogen and plasminogen activators that are not tightly bound to polymerizing fibrin (Braaten *et al*, 1994). Platelet-bound fibrin has been found to display 20-50% greater resistance to lysis than non platelet-bound fibrin. Synthetic peptides modelled on the GPIIb-IIIa-fibrinogen recognition sequences have been shown to inhibit platelet aggregation through inhibition of adhesive interactions between fibrinogen and the platelet and subsequently acceleration of clot lysis at the platelet/fibrin interface occurs (Braaten *et al*, 1994). Recently small peptides containing RGD sequences and rich in disulfide bridges called disintegrins, because of their ability to inhibit numerous adhesive interactions, have been isolated from viper snake venoms (Shebuski *et al*, 1989). These peptides bind with high affinity to the GPIIb-IIIa receptor inhibiting

platelet aggregation (Huang *et al*, 1991). The conformation of the RGD amino acid sequence within the disintegrins accounts for the fact that they are 1000 times more potent than linear RGD-containing peptides in inhibiting platelet aggregation. Several studies have indicated that these snake venom-derived peptides may be useful antithrombotic agents (Cheng *et al*, 1994). However, this may be too simplistic a view as evidence is emerging that fibrinogen contains multiple domains that contribute to the expression of full platelet binding activity, in addition to the RGD and dodecapeptide sequences previously described (Peerschke and Galanakis, 1996). Components released from constituent cells also influence clot susceptibility to lysis. Platelet release of PAI-1 and α_2-antiplasmin, which becomes crosslinked to fibrin through the action of factor XIII, are inhibitors within the clot making it resistant to plasmic lysis (Braaten *et al*, 1993; Sakata and Aoki, 1980).

We have shown in the previous chapter that neutrophils possess fibrin solubilizing activity, with neutrophil membrane proteases effecting clot lysis in the absence of stimuli and when these cells are stimulated with a low dose of PMA that does not cause lysosomal enzyme release. This process generates products that are distinctly different in molecular weight from those produced during plasmic or lysosomal enzyme lysis of fibrin. The results also suggested that this neutrophil activity might modify the resistance of platelet-rich clots to lysis by plasmin.

This study monitors the effect of neutrophils on plasmic clot lysis when platelets are incorporated into the clot. When the role played by fibrin(ogen) degradation products in modulating plasmic clot lysis is considered, it is possible that the products generated by neutrophil membrane-mediated clot lysis, may influence plasmic clot lysis in the presence of platelets.

Results

Plasmin-mediated fibrinolysis: modification by the presence of platelets and neutrophils

Thrombin (0.4 units/ml final concentration) was added to a mixture of ^{125}I-labelled fibrinogen (125 μg), free of any contaminating plasminogen, $2x10^8$ washed platelets and urokinase (50 IU/ml final concentration) in a final volume of 500 μl with or without $2.5x10^6$ neutrophils. Subsequent to macroscopically visible clot formation, pure human plasminogen (20 μg/ml final concentration) was added to initiate clot lysis. Lysis at 60 min, as measured by release of ^{125}I-labelled fibrin into the supernatant, was compared to plasmic lysis in the absence of platelets and neutrophils. The results are presented in Table 1. Less than 1% of the fibrin was solubilized in the absence of proteases. Plasmic lysis releases 98 μg soluble fibrin products in 60 min. The presence of platelets inhibited plasmic clot lysis by 50%. This inhibition was partially overcome by the addition of neutrophils such that plasmic lysis was now inhibited by 26% (Table 1).

Table 1. Plasmic degradation of ^{125}I-labelled fibrin clot at 60 min with modification by incorporated platelets and incorporated platelets together with PMA-stimulated neutrophils ($2.5x10^6$ neutrophils/$2x10^8$ platelets/125 μg ^{125}I-labelled fibrin clot in a final volume of 500 μl). Each point is the mean of triplicates that did not differ by more than 2% and the illustrated results are one of three replicate experiments.

Constituents of ^{125}I-labelled fibrin clot	^{125}I-labelled fibrin released (μg)	% inhibition of plasmin-mediated fibrin degradation
urokinase/plasminogen	98	0
urokinase/plasminogen + platelets	49	50
urokinase/plasminogen + platelets + neutrophils	72	26

Modification of platelet function by fibrin degradation products generated during fibrin lysis by neutrophil membrane proteolytic activity

Since the inhibition of plasmic lysis in the presence of platelets was partially overcome by the proteolytic activity of neutrophils, experiments were performed to investigate the effect of the neutrophil-derived FDP's on platelet function.

Platelet aggregation

Washed platelets ($2x10^8$ platelets/ml) were preincubated (10 min, 37°C) with FDP's or the low molecular weight fibrin peptides or the corresponding controls (see methods), generated during fibrin lysis by neutrophils. Platelet aggregation, measured using a platelet aggregometer (Chronolog Corporation, Havertown PA, USA), was initiated by the addition of thrombin (0.05 units/ml final concentration). The effect of the entire mix of FDP's on platelet aggregation could not be interpreted as the traces representative of platelet aggregation in the presence of the FDP's indicated evidence of spontaneous aggregation without the requirement for addition of exogenous thrombin. Modulation of platelet aggregation by the low molecular weight fibrin peptides is indicated in Fig 1. The peptides show a concentration dependent inhibition of platelet aggregation. A concentration of 75 μg peptides produced 40% inhibition of aggregation while the presence of 150 μg of peptides increased this inhibition to 60%.

Clot retraction

Washed platelets ($2x10^8$ platelets) were preincubated (15 min, 37°C) with FDP's, or the low molecular weight fibrin peptides or the corresponding controls (see methods), generated during fibrin lysis by neutrophils, or echistatin used as a positive control (1 μM final concentration). Echistatin is a snake venom-derived small RGD-containing protein (Mr 5.4 kDa) which inhibits platelet aggregation through its ability to bind to the GPIIb-IIIa receptor (Garsky *et al*, 1989). Plasminogen free fibrinogen (250 μg) was added and the volume adjusted to 500 μl with HBSS. Clot formation was initiated by the addition of thrombin (0.4 units/ml final concentration). The resulting clot retraction following clot formation was observed macroscopically and a photographic record kept over a period of 60 min as shown in Fig 2. Complete inhibition of clot retraction was observed in the presence of echistatin. No inhibition of clot retraction in the presence of the FDP's, or the low molecular weight fibrin peptides or the corresponding controls, generated during fibrin lysis by neutrophils was observed.

Figure 1. Platelet aggregation in response to thrombin stimulation of platelets. Platelets were used alone or following preincubation with peptides obtained from neutrophil conditioned medium solubilization of fibin clot ($2x10^8$ platelets/0.75 or 1.5 mg peptides in a final volume of 500 µl). An equivalent peptide control preincubated with platelets showed no inhibition in platelet aggregation compared to platelets alone (results not shown). The results illustrated are representative of one of four replicate experiments where the percentage inhibition of platelet aggregation produced by peptides did not differ by more than 8% for each concentration used.

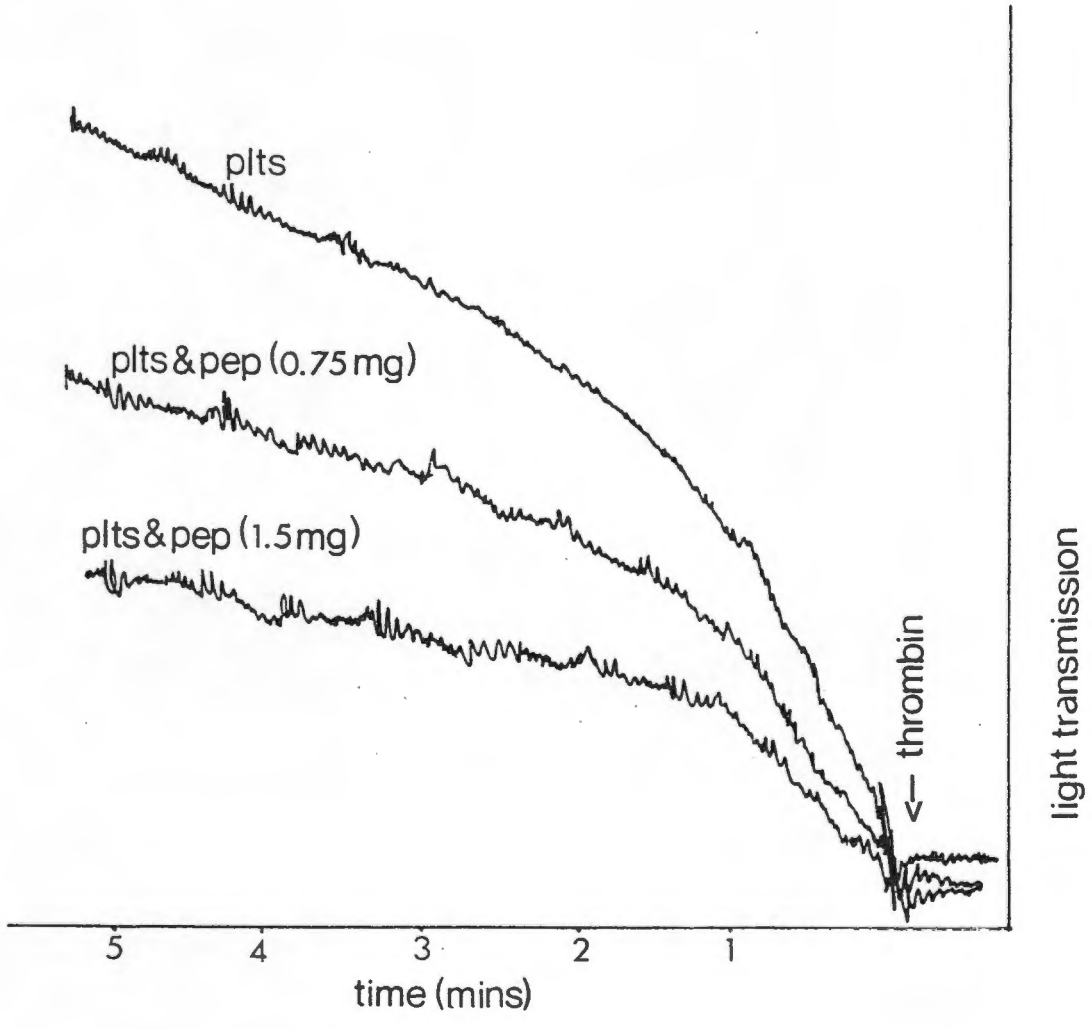
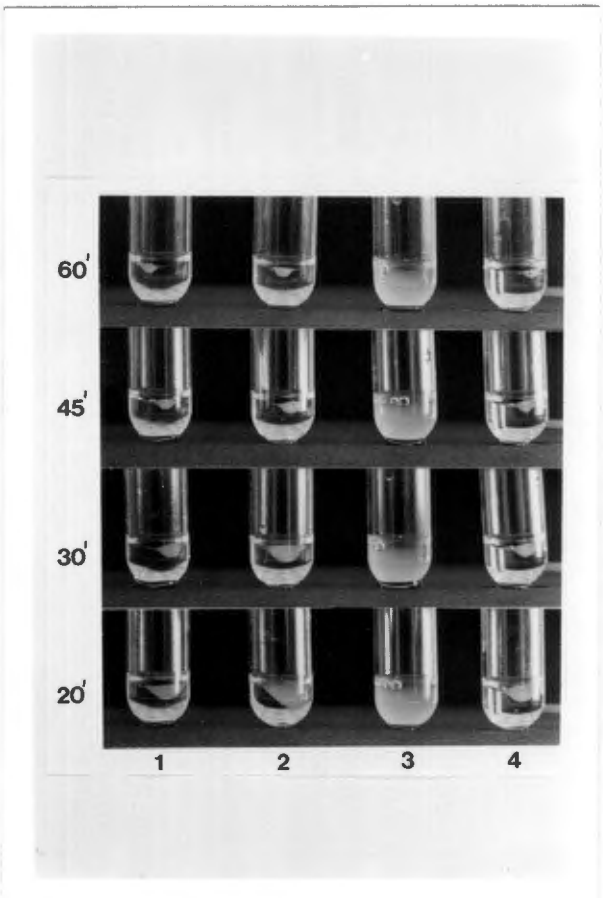


Figure 2. Fibrin clot retraction at 20 min, 30 min, 45 min, 60 min in the presence of platelets alone (Track 1); platelets and peptides derived from neutrophil conditioned medium degradation of fibrin clot (Track 2); platelets and echistatin (Track 3); platelets and peptide control (Track 4). The experiment was repeated three times with the same results obtained on each occassion.



Plasmic fibrin lysis in the presence of platelets: modification by peptides produced by neutrophil membrane lysis of fibrin

Plasminogen free ^{125}I-labelled fibrinogen (250 μg) was added to washed platelets ($2x10^8$ platelets) that had been preincubated (15 min, 37°C) with 1.5 mg FDP's, or 1.5 mg of the low molecular weight fibrin peptides or the corresponding controls (see methods), generated during fibrin lysis by neutrophils. Clot formation was initiated by the addition of thrombin (0.4 units/ml final concentration). Following an incubation period of 25 min, which allowed clot formation and retraction to occur, clot lysis was commenced by the addition of urokinase (50 IU/ml final concentration) and plasminogen (20 μg/ml final concentration). Plasmic lysis of fibrin in the absence of platelets and the FDP's served as a control. Clot lysis was monitored as the release of ^{125}I-labelled fibrin products into the supernatant at 60 min.

No modulation of plasmic lysis of fibrin was detected when the platelets were preincubated with the low molecular weight fibrin peptides (Table 2). The inhibition of plasmic lysis observed in the presence of platelets was partially overcome when the platelets were preincubated with the FDP's. This inhibition was due to the high molecular weight fibrin products as a similar result to that illustrated in Table 2 was obtained when the products were used after removal of the low molecular weight fibrin peptides by dialysis.

Table 2. Plasmic degradation of ^{125}I-labelled fibrin clot (250 μg) at 60 min and modification by incorporated platelets, alone or preincubated with FDP, FDP control, a peptide control or peptides derived from neutrophil conditioned medium solubilization of fibrin clot (1.5 mg FDP or peptides/$2x10^8$ platelets/250 μg ^{125}I-labelled fibrin clot in a final volume of 1 ml). Each value is the mean of duplicates which did not differ by more than 10% and four experiments gave results within 10% of these.

Constituents of ^{125}I-labelled fibrin clot	^{125}I-labelled fibrin released (μg)	% inhibition of plasmin-mediated fibrinolysis
urokinase/plasminogen	185	0
urokinase/plasminogen + platelets	60	68
urokinase/plasminogen + platelets + FDP	107	42
urokinase/plasminogen + platelets + FDP control	65	65
urokinase/plasminogen + platelets + peptides	55	70
urokinase/plasminogen + platelets + peptide control	55	70

Discussion

From the results presented in this chapter it appears that platelet-mediated inhibition of plasmic lysis can be overcome, in part, by the presence of neutrophils in the clot. *In vivo*, neutrophils become incorporated into the forming clot via a dynamic process involving neutrophil membrane integrin receptors and platelet expressed P-selectin (Palabrica *et al*, 1992). Other factors, for example the use of thrombolytic therapy, may also influence the number of neutrophils at the site of tissue damage and thus associated with the clot. The infusion of streptokinase has been shown to result in a significant increase in the number of circulating neutrophils (Adams *et al*, 1995; Ranjadayalan *et al*, 1991). Thus *in vivo*, thrombolytic therapy with plasminogen activators may proceed via both the generation of plasmin and a secondary process involving an increase in the neutrophil content of the thrombus. This would be in line with the proposal by Langleben *et al* (1990) that success of thrombolytic therapy is not only due to plasmic lysis but also in part to intrinsic cellular activity. The cellular component of the fibrinolytic process may well become important in clot lysis when inhibition of plasmic lysis occurs through the release of PAI-1 from platelets. The mechanism of the neutrophil-mediated reversal of plasmic clot lysis in the presence of platelets observed is not clear. It could be due to the intrinsic fibrinolytic activity of the neutrophil and/or inactivation of PAI-1 directly by the proteolytic activity of the neutrophil membrane. Studies have shown that the neutrophil serine protease, elastase, can inactivate PAI-1 (Wu *et al*, 1995). The protease within the neutrophil membrane responsible for fibrin lysis, under conditions of non-release of lysosomal enzymes, is a serine protease. A small but consistent increase in plasmic lysis in the presence of neutrophil conditioned medium (ie FDP control) and platelets is observed indicating possible inactivation of PAI-1 by the membrane enzyme content of the neutrophil conditioned medium. Results presented in the previous chapter indicate that considerable amounts of this protease are released from the membrane in the presence of fibrin. Thus this proteolytic activity may well inactivate PAI-1 (although this study did not address this point) and reverse platelet induced inhibition of plasmic clot lysis when intact neutrophils are present in the clot together with platelets.

FDP's have been demonstrated to possess biological activity, particularly with regard to platelet function (Peerschke and Galanakis, 1996; Kloczewiak *et al*, 1982; Plow *et al*, 1987; Du *et al*, 1991). The interaction of platelets with one another after initial adhesion to a thrombogenic substrate is fundamental to the formation of thrombus. It is possible therefore that the fibrin products generated during neutrophil membrane-mediated clot lysis will alter plasmic lysis in the

presence of platelets, through modulation of the interaction of fibrinogen with the platelet. In particular degradation products containing the RGD or KQAGDV sequences have been shown to inhibit platelet aggregation and clot retraction. Uncoupling of the interaction of fibrin binding to the platelet GPIIb-IIIa receptor by a potent antiadhesive peptide D-RGDW sequence, has also been demonstrated to enhance plasmic solubilization of fibrin at the platelet surface (Braaten *et al*, 1994). In addition to the well described platelet recognition sequences Peerschke and Galanakis (1996) have shown evidence for divalent cation dependent and independent platelet interactions with novel, non-RGD, non-dodecapeptide containing platelet recognition sequences in plasmin-derived FDP's D and E and it has been suggested that RGD sites may not figure prominently in fibrinogen binding (Farrell *et al*, 1992).

The low molecular weight fibrin peptides generated during clot lysis by the activity of the neutrophil membrane were able to inhibit platelet aggregation but did not affect clot retraction. It has been demonstrated that sequences within fibrin that inhibit platelet aggregation may not affect clot retraction (Cohen *et al*, 1989). Recently evidence was presented by Rooney *et al* (1996) that the γ chain C-terminus of fibrin(ogen) is essential for platelet aggregation but not for clot retraction. This distinct action for regions within fibrinogen might well explain why the low molecular weight fibrin peptides inhibit platelet aggregation but do not influence clot lysis. The inhibition of platelet aggregation by the fibrin peptides indicates interference by the peptides with the interaction of fibrinogen with the platelet surface. This appears to be insufficient to effect alteration of plasmic fibrin solubilization in the presence of platelets. The lack of ability of these fibrin peptides to enhance plasmic lysis of platelet containing clots might be related to their inability to modulate clot retraction. This is a process that compacts the fibrin bound to the surface of the platelet and prevents the diffusion of plasminogen activators and plasminogen into the clot matrix (Kunitada *et al*, 1992). It is characterized by the alignment of fibrin fibres following binding of polymerizing fibrinogen to the platelet receptor and in doing so determines clot tension and creates areas of high density at the surface of the platelet (Chao *et al*, 1976; Braaten *et al*, 1994). Inhibition of clot retraction is associated with reduction in the resistance of platelet rich clots to plasmic lysis (Kunitada *et al*, 1992).

In contrast to the effect of the low molecular weight FDP's on plasmic clot lysis, the high molecular weight fibrin products were able to partially overcome the resistance of plasmic clot lysis in the presence of platelets. The mechanism for this is not clear as these products did not inhibit clot retraction. Even though the effect of these products on platelet aggregation could not

be determined in this study it is possible that they do inhibit platelet aggregation, which in turn could alter plasmic clot lysis in the presence of platelets. Their effect on platelet aggregation could not be determined as they caused "spontaneous" platelet aggregation, which is most probably the result of active thrombin contaminating these products. This arises through the irreversible binding of thrombin to fibrinogen during the initial clot formation process, which would remain in an active form bound to the resulting FDP's (Weitz *et al*, 1990; Francis *et al*, 1983) and would have the capacity to stimulate platelets preincubated with an FDP mix. The preparative process of peptide formation in the experiments involved the addition of TCA, ether extraction and washes with TFA (see Appendix 1: Methods), all of which may have served to inactivate any bound thrombin bound to fibrin peptides. Thus spontaneous platelet aggregation before the addition of exogenous thrombin did not present a problem in the platelet aggregation studies involving peptides.

From the results presented in this study it may be concluded that the products generated by lysis of fibrin by a neutrophil membrane serine protease have several beneficial effects during inflammation. The significant inhibition of platelet aggregation by peptides formed as a result of neutrophil-mediated fibrinolysis may represent an important negative feedback system operative *in vivo*. Following effective thrombus formation and arrest of haemorrhage subsequent cellular fibrinolytic activity releases products which prevent further platelet aggregation and clot formation. However, recanalization, localization of the clot to the site of tissue injury and approximation of the edges of the wound is still allowed to proceed as these products do not modulate the retraction process. In addition the reversal of the resistance to plasmic degradation, usually seen in the presence of platelets, by release of neutrophil-derived fibrin products would also contribute to an increased rate of fibrinolysis and recanalization.

CHAPTER 7

Digestion of ^{125}I-labelled plasmin-derived fibrin degradation products by neutrophil lysosomal enzymes

CHAPTER 7

Digestion of ^{125}I-labelled plasmin-derived fibrin degradation products by neutrophil lysosomal enzymes

Introduction

Plasmin-mediated fibrinolysis, which yields major core products D-dimer (184 kDa), E (41 kDa), D (83-100 kDa) as well as other lower molecular weight degradation products (Pizzo *et al*, 1973), is considered a major pathway for solubilization of fibrin. However, nonplasmin-mediated pathways of fibrinolysis and in particular those mediated by neutrophils are recognized as being important for clot solubilization. The accumulation of neutrophils at sites of inflammation and vascular injury where fibrin deposition occurs is a dynamic process involving specific neutrophil receptors (Palabrica *et al*, 1992; Wright *et al*, 1988; Altieri *et al*, 1993). This interaction with and adherence of neutrophils to extracellular matrix proteins can result in cellular activation and release of lysosomal enzymes (Nathan *et al*, 1989, Liles *et al*, 1995) which contribute to fibrinolysis. Thus the products of plasmin-mediated fibrinolysis and neutrophil lysosomal enzymes are in close proximity *in vivo*. This may be particularly relevant clinically in situations where patients are receiving infusions of thrombolytic agents. Infusion of one such agent, streptokinase, results in a significant increase in the number of circulating neutrophils (Adams *et al*, 1995; Ranjadayalan *et al*, 1991) together with a rise in circulating levels of HNE and neutrophil-elastase-derived fibrinopeptide $B\beta_{30-43}$ (Ranjadayalan *et al*, 1991), implying the presence of activated neutrophils.

Neutrophil lysosomal enzyme lysis of fibrin has been ascribed to elastase and cathepsin G (Moroz, 1984) and yields distinctly different products from neutrophil membrane-associated and plasmic lysis of fibrin (Chapter 5; Plow and Edgington, 1975; Plow *et al*, 1983; Francis and Marder, 1986). Neutrophil lysosomal enzymes could possibly provide a valuable direct alternative to plasmic fibrin solubilization if released in high concentration or indirectly through elastase conversion of plasminogen to miniplasminogen (Kolev *et al*, 1996). The further importance of elastase to the process of clot lysis is recognised through its ability to inactivate plasmin inhibitor which becomes incorporated into fibrin clots during *in vivo* clot formation (Wu *et al*, 1995). Two

recent studies have shown that elastase is able to degrade D-dimer (Bach-Gansmo *et al*, 1996) and other plasmin-derived FDP's (Leavell *et al*, 1996). This secondary digestion of plasmin-derived FDP's produced molecules which were more potent chemoattractants than native plasmin FDP's (Leavell *et al*, 1996).

The aim of this study was to investigate the ability of neutrophil lysosomal enzymes to further degrade the end products of plasmic fibrin degradation and to characterize the enzymatic and biochemical processes involved in this pathway.

Results

Degradation of plasmic ^{125}I-labelled fibrin products with neutrophil lysosomal enzymes

Degradation of ^{125}I-labelled fibrin with plasmin generated major degradation products that migrated on SDS-PAGE, in the absence of reducing conditions, to apparent molecular weights of 182 kDa (probable D dimer), 130 kDa, 98 kDa (probable D fragment), 52 kDa, 42 kDa (probable fragment E), 36 kDa and a number of fragments <30 kDa (Fig 1). The products with apparent molecular weights of 182 kDa, 130 kDa, 98 kDa, 52 kDa and 42 kDa were degraded within 30 min by neutrophil lysosomal enzymes ($5x10^6$ cell equivalents) (Fig 1). Further incubation of the reaction mixture, once these degradation products had formed, lead to the generation of high molecular weight products of 182 kDa and 120 kDa which were not sensitive to the further addition of plasmin (Fig 1). The addition of a fresh preparation of lysosomal enzymes led to repeated degradation and reformation of these high molecular weight products (Fig 1). A decrease in the time of the initial degradation of the plasmin-derived degradation products and an increase in the time required for the formation of the high molecular weight products was observed as the concentration of lysosomal enzyme was increased (data not shown). Removal of plasminogen from the plasmin-derived ^{125}I-labelled fibrin degradation products by lysine sepharose affinity chromatography did not prevent the production of high molecular weight products during degradation with lysosomal enzymes (data not shown).

Incubation of the plasmin-derived ^{125}I-labelled fibrin degradation products with pure human neutrophil elastase resulted in rapid (within 30 min) degradation of the products with apparent molecular weights of 182 kDa, 130 kDa, 52 kDa and 42 kDa, while the product with an apparent molecular weight of 98 kDa was more resistant to elastase activity (Fig 1). In contrast, although pure human neutrophil cathepsin G was able to effect degradation of ^{125}I-labelled fibrin products with apparent molecular weights of 98 kDa, 52 kDa, 42 kDa and <30 kDa, D-dimer was completely resistant and the 130 kDa product relatively resistant to cathepsin G activity (Fig 1). No high molecular weight products were formed during a 24 h incubation period of these ^{125}I-labelled fibrin degradation products with elastase or cathepsin G (data not shown).

Effect of inhibitors on the degradation of plasmin-derived ^{125}I-labelled fibrin degradation products by neutrophil lysosomal enzymes

The inhibitors TPCK, TLCK, E64 and a specific cathepsin G inhibitor did not inhibit either lysosomal enzyme degradation of the plasmic fibrin degradation products or the production of the lysosomal enzyme-derived high molecular weight ^{125}I-labelled fibrin degradation products (data not shown). However, the serine protease inhibitors AEBSF and PMSF completely inhibited this degradation (Fig 2) while inhibition of degradation of the 182 kDa product was observed in the presence of the specific elastase inhibitor MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (Fig 2).

Although no inhibition of degradation of the plasmin-derived ^{125}I-labelled fibrin degradation products by neutrophil lysosomal enzymes occurred in the presence of EDTA or either spermine (100 mM) or spermidine (100 mM), primary amines that inhibit factor XIII transglutaminase activity, the subsequent formation of high molecular weight degradation products did not occur (Fig 2). Spermine or spermidine at a concentration of 10 mM delayed the production of the lysosomal-enzyme derived high molecular weight degradation products, with initiation of reformation in the absence of inhibitor occurring at 90 min but in the presence of 10 mM inhibitor reformation began at 5 hr (data not shown).

Figure 1 SDS-PAGE analysis (10% non-reduced) of the ^{125}I-labelled protein in solution: fibrinogen standard (track 1); products resulting from full solubilization of fibrin clot (250 μg) by the addition of urokinase (40 IU/ml) and human plasminogen (40 μg/ml) (track 2); products generated on addition of neutrophil lysosomal enzyme ($5x10^6$ cell equivalents) to plasmin-derived fibrin products shown in track 2 for 30 min, 60 min, 90 min, 3 h, 24 h (tracks 3-8); nature of products generated on further addition of neutrophil lysosomal enzyme ($5x10^6$ cell equivalents) to the products in track 8 (24 h neutrophil lysosomal enzyme digest of plasmic fibrin digest) at 30 min and 24 h (tracks 9 and 10); addition of human plasminogen (40 μg/ml) and urokinase (40 IU/ml) to the products in track 8 (24 h neutrophil lysosomal enzyme digest of plasmic fibrin digest) (track 11), products generated by addition of pure human neutrophil elastase (25 μg/mg of protein) to plasmin-derived fibrin products shown in track 2 at 3 h and 24 h (tracks 12 and 13), products generated by addition of pure human leukocyte cathepsin G (25 μg/mg of protein) to plasmin-derived fibrin products shown in track 2 at 3 h and 24 h (tracks 14 and 15). Molecular weight markers as indicated.

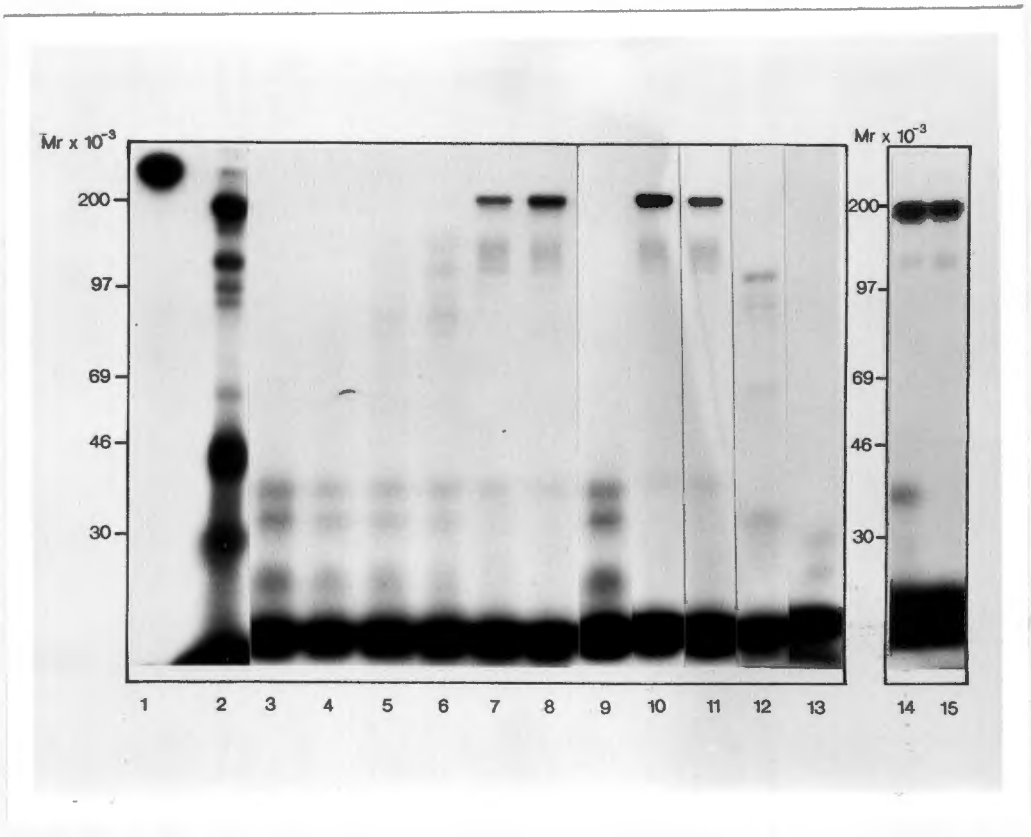
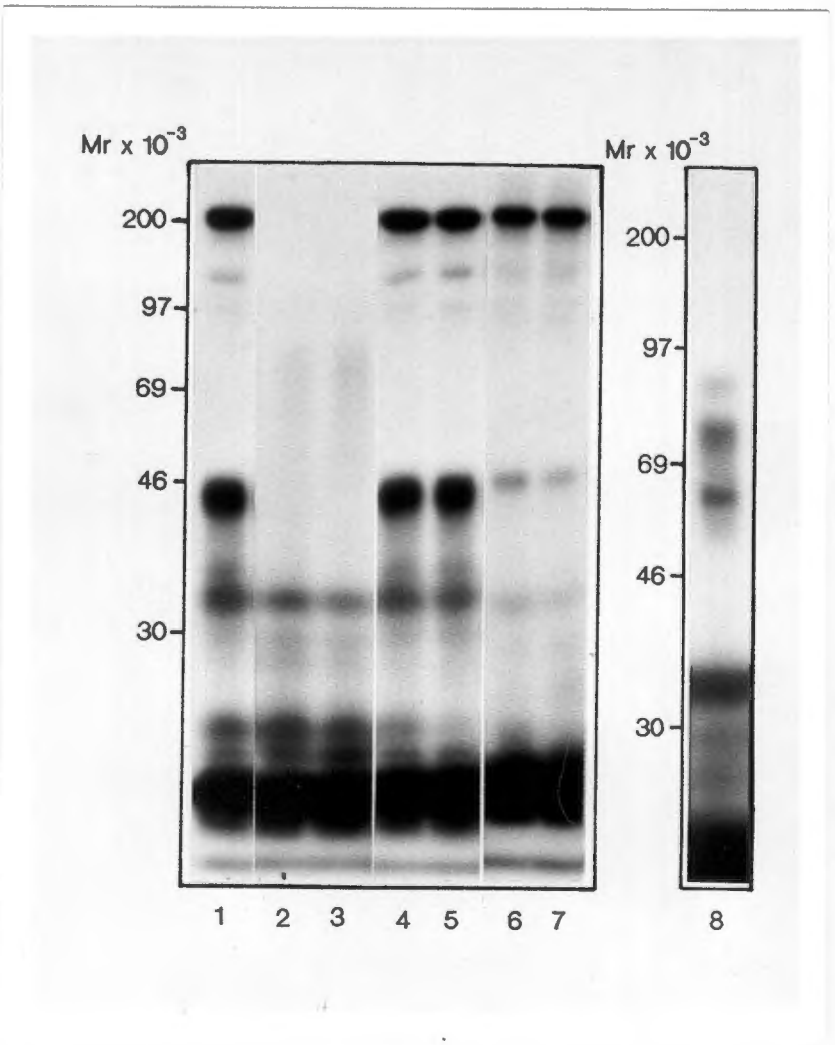


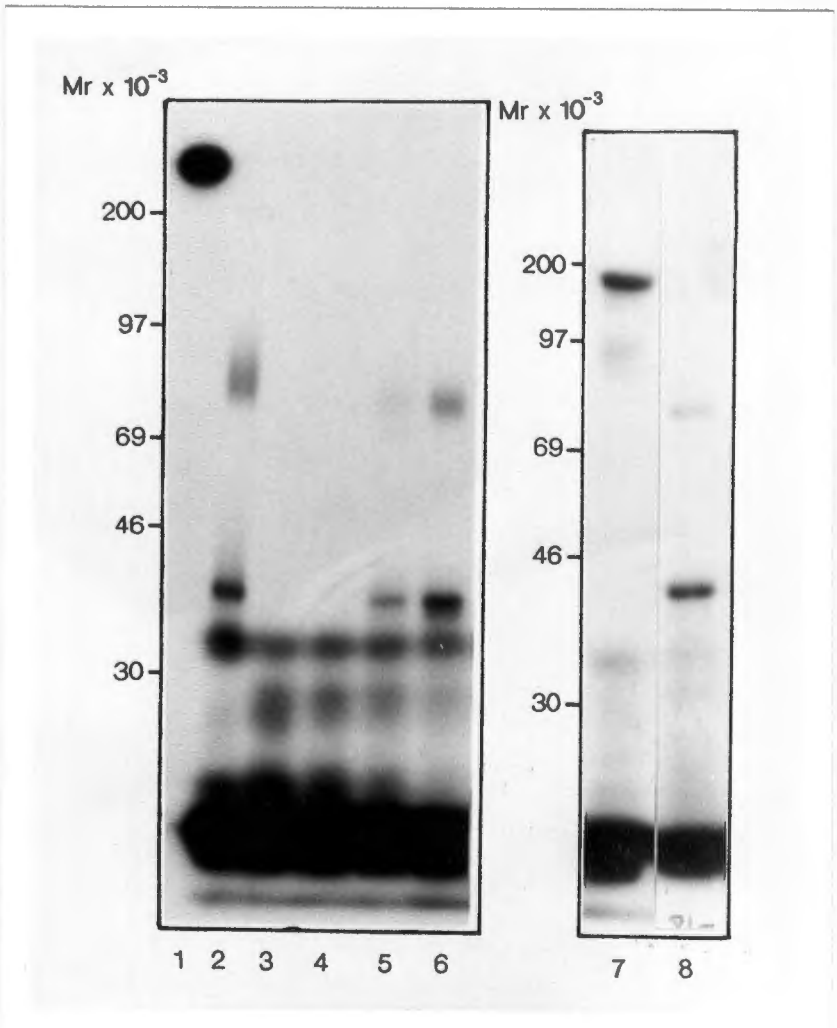
Figure 2 SDS-PAGE analysis (10% non-reduced) of the ^{125}I-labelled protein in solution: on full solubilization of fibrin clot (250 μg) by the addition of urokinase (40 IU/ml) and human plasminogen (40 μg/ml) (track 1); addition of neutrophil lysosomal enzyme ($5x10^6$ cell equivalents) to plasmin-derived products shown in track 1 at 90 min with no inhibitor (track 2), in the presence of spermine (100 mM) at 90 min (track 3), in the presence of PMSF (1 mM) at 90 min (track 4), in the presence of AEBSF (1 mM) at 90 min (track 5), in the presence of MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (10 μM) at 90 min and 3 h (tracks 6 and 7), in the presence of EDTA (10 mM) at 90 min (track 8). Molecular weight markers as indicated.



Inhibition of disulfide bonding

Incubation of the ^{125}I-labelled FDP's with neutrophil lysosomal enzymes in the presence of a reducing agent did not prevent the formation of high molecular weight ^{125}I-labelled fibrin-derived products (Fig 3).

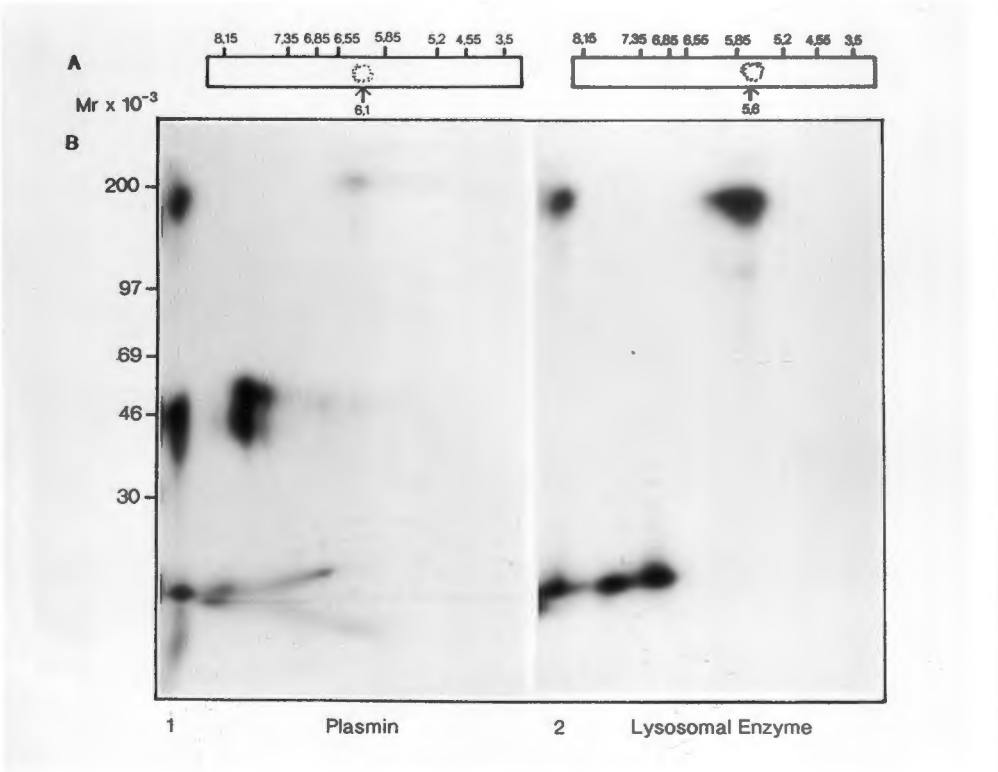
Figure 3 SDS-PAGE analysis (10%) of ^{125}I-labelled protein in solution: fibrinogen standard (track1); products resulting from full solubilization of fibrin clot (250 μg) by the addition of urokinase (40 IU/ml) and human plasminogen (40 μg/ml) in the presence of dithiothreitol (1 mM) (track 2) followed by addition of neutrophil lysosomal enzyme ($5x10^6$ cell equivalents) to plasmin-derived fibrin products shown in track 2 at 30 min, 60 min, 90 min, 8 h and 24 h (tracks 3-6); nature of products generated on addition of neutrophil lysosomal enzyme ($5x10^6$ cell equivalents) to plasmin-derived fibrin products at 24 h run under non-reducing (track 7) and reducing conditions (track 8). Molecular weight markers as indicated.



Iso-electric point of the lysosomal enzyme-derived high molecular weight ^{125}I-labelled fibrin degradation products

Two dimensional iso-electrophoresis determined the pI of the 182 kDa lysosomal enzyme-derived high molecular weight ^{125}I-labelled FDP to be 5.6 while the pI of the plasmin-derived product with this molecular weight was 6.1 (Fig 4).

Figure 4 Iso-electric focusing of ^{125}I-labelled fibrin degradation products produced after full solubilization of fibrin clot by plasmin and by the addition of neutrophil lysosomal enzyme to these products, followed by SDS-PAGE. A. ^{125}I-labelled fibrin degradation products produced by plasmic digestion of fibrin clot and by the addition of neutrophil lysosomal enzyme to these products were analysed on an isoelectric PAG plate with a broad pH range as indicated. **B.** The products separated on the isoelectric PAG plates (A) were then subjected to SDS-PAGE (10% non-reduced) followed by autoradiography. The product of apparent molecular weight 182 kDa generated by plasmin and neutrophil lysosomal enzyme and the position to which they migrated in the first dimension, corresponding to isoelectric points 5.6 and 6.1, are indicated with arrows. Track 1 - plasmic digestion products; track 2 - products following addition of neutrophil lysosomal enzyme.

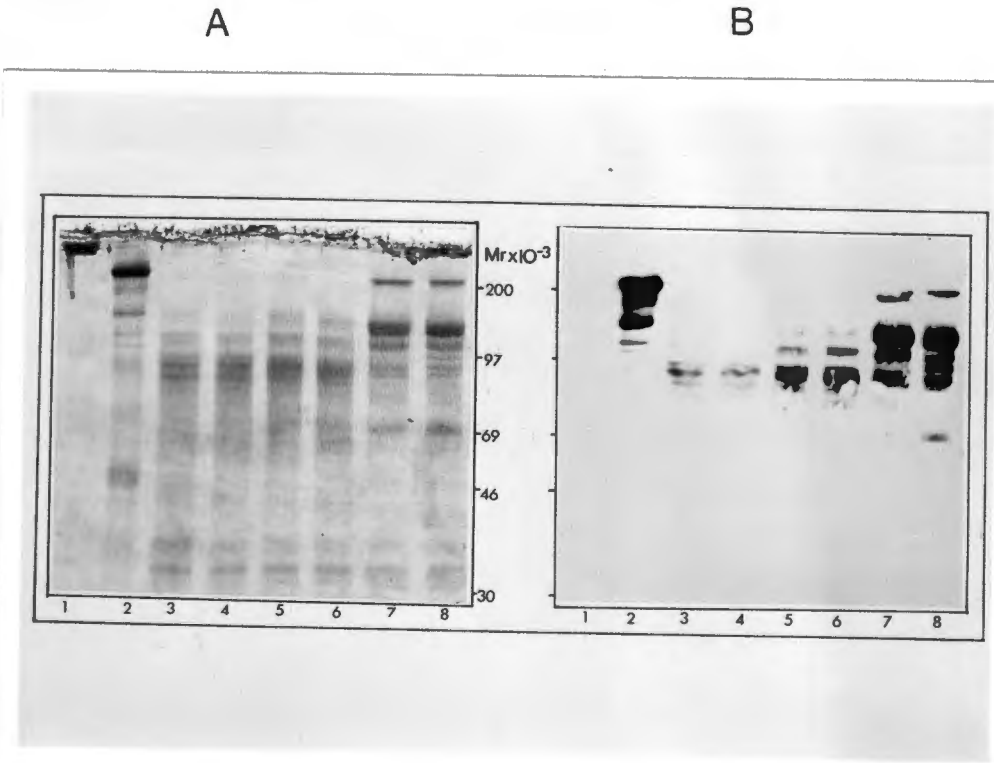


Western blotting using monoclonal antibody directed against D-dimer

Plasmin-derived FDP's were incubated with a neutrophil lysosomal enzyme preparation (37°C) and sampling was performed over a period of 24 hr. These samples were then subjected to SDS-PAGE (10% non-reduced). The separated proteins were transferred to an Immobilon-P membrane via a western blotting technique (Towbin *et al*, 1979) and probed with a monoclonal antibody directed against D-dimer, which shows no reactivity with fibrin monomer. The results are shown in Fig 5. The anti-D-dimer antibody did not recognize fibrinogen. However, it showed strong reactivity with material running at appparent molecular weight >90 kDa in the plasmic fibrin digest as well as the products >80 kDa that are reformed when the plasmic products are incubated with lysosomal enzymes. A control experiment, where no primary antibody was used, showed no non-specific binding of the second antibody-peroxidase conjugate (data not shown).

Figure 5 SDS-PAGE analysis (10% non-reduced) of a plasmic digest of fibrin and lysosomal enzyme digestion of the plasmin products (**A**) and probing of the products with monoclonal antibody directed against D-dimer (**B**).

Fibrinogen standard (track 1); full solubilization of fibrin clot (250 μg) by the addition of urokinase (40 IU/ml) and human plasminogen (40 ug/ml) (track 2); addition of neutrophil lysosomal enzyme ($5x10^6$ cell equilavents) to plasmic fibrin digest at 30 min, 60 min, 90 min, 3 hr, 8 hr, 24 hr (tracks 3-8).



Discussion

From the results presented it is evident that lysosomal enzymes are able to rapidly degrade plasmin-derived fibrin products, in particular those products with molecular weights consisitent with D-dimer and fragment E, resulting in a corresponding increase in the concentration of products of lower molecular weight. With time, however, there is reformation of high molecular weight ^{125}I-labelled fibrin products. The rate at which these products are reformed depended on the concentration of lysosomal enzyme used, with more rapid degradation occurring at high concentrations but subsequent slower reformation. The high molecular weight products formed during lysosomal enzyme degradation are insensitive to cleavage by plasmin but the further addition of lysosomal enzyme allowed repeated breakdown and subsequent reformation. Characterisation of the lysosomal enzymes involved in the initial degradation of the plasmic products revealed them to be sensitive to serine protease inhibitors, AEBSF and PMSF and the elastase inhibitor MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl. The formation of the high molecular weight products could not be assessed in the presence of elastase inhibitors, as they prevented breakdown of plasmin products, but when using pure human neutrophil elastase as the enzyme for plasmic product degradation no subsequent reformation occurred. In the presence of 10 mM EDTA lysosomal enzyme-mediated degradation of plasmic products occurred but no subsequent reformation was observed, implying a calcium dependent process. Disulfide bonding is present within the reformed material as performing the experiment or running the SDS-PAGE under reducing conditions lowers the apparent molecular weight of the reformed products.

The pI of the reformed product, with an apparent molecular weight of 182 kDa which is identical to the apparent molecular weight of a plasmic product (probable D-dimer) was different. However, a monoclonal antibody directed against D-dimer and therefore specific for crosslinked material, recognizes the high molecular weight products produced over time, following addition of neutrophil lysosomal enzyme to plasmin-derived FDP's.

Plasminogen binds selectively to fibrin allowing for generation of plasmin activity at the clot surface (Fears, 1989; Kaczmarek *et al*, 1993) and remains bound to products following digestion. Cross-linked complexes from dissolved clot that have been observed by electron microscopy reveal plasminogen bound to the end of fibrin or bridging the ends of two fibrin molecules with the formation of larger complexes (Weisel *et al*, 1994). However, plasminogen association with FDP's does not appear to be playing a role in the reformation process described in this study as its removal from the degradation products by lysine sepharose affinity chromatography did not affect

the production of high molecular weight material following addition of neutrophil lysosomal enzymes.

Factor XIII is a calcium dependent enzyme, which on activation by thrombin is able to catalyse a reaction that links the glutamyl portion of a glutamine residue with a lysine side chain. This enzyme is bound as an $\alpha_2\beta_2$ tetramer to the D domain of fibrinogen (Greenberg *et al*, 1982; Mary *et al*, 1987) and would therefore be expected to be bound to plasmic FDP's in an active form as thrombin was used to form the initial clot. Fibrin clot is able to act as a reservoir for enzymatically active thrombin which is resistant to inactivation by circulating thrombin inhibitors (Weitz *et al*, 1990). During plasmin-mediated fibrinolysis soluble FDP's are released into the circulation, with active thrombin complexed to these products (Francis *et al*, 1983). Plasmin is neither able to activate or degrade factor XIII (Rider and McDonagh, 1981) but several other enzymes besides thrombin are also able to activate factor XIII. These include trypsin and papain (Schwartz *et al*, 1973; Conery and Berliner, 1983) and other plasma serine proteases, factor Xa and kallikrein (Folk and Finlayson, 1977; McDonagh and McDonagh, 1975; Laudano *et al*, 1983). It is tenable, therefore, that constituents of a neutrophil lysosomal enzyme preparation may be able to convert factor XIII to its active form by cleavage of the α-subunit Arg_{37}-Gly_{38} bond (Hornyak *et al*, 1989). Thereafter, the presence of calcium ions allows β subunits to dissociate from the cleaved zymogen to yield catalytically active factor XIIIa (Chung *et al*, 1974). The presence of factor XIII inhibitors, spermine and spermidine, was able to significantly retard the reformation of high molecular weight fibrin products and at a concentration of 100 mM completely inhibit it. In the presence of EDTA, which would render this calcium dependent enzyme inactive, this process was completely abolished.

The various products produced by plasmic activity on fibrin have been shown to have immunomodulatory effects. These include downregulation of neutrophil oxidative metabolism (Kazura *et al*, 1989), modulation of neutrophil/endothelial cell interactions (Fischer *et al*, 1991) and neutrophil chemotaxis (Kazura *et al*, 1989). In particular, fragment E has been shown to be a potent source of angiogenic activity (Thompson *et al*, 1992) and may be important in producing focal smooth-muscle cell proliferation (Strirk *et al*, 1993), a key event in the formation of stenosing atherosclerotic lesions. This study shows that neutrophils are able to rapidly degrade these plasmin-derived FDP's which may have important biological consequences *in vivo*. It has been suggested that circulating levels of D-dimer may be suitable as a laboratory marker of deep venous thrombosis (Declerck *et al*, 1987), pulmonary embolism (Bounameaux *et al*, 1990) and

for monitoring thrombolysis in patients with acute myocardial infarction (Lew *et al*, 1986), although this remains controversial (Francis *et al*, 1986; Jude *et al*, 1992). In addition, Lassila *et al* (1993) found a significant association between the severity of peripheral arterial occlusive disease and the levels of circulating D-dimer.

Bangalore and Travis (1994) and Owens *et al* (1995a) showed that the neutrophil lysosomal enzymes elastase and cathepsin G rebind to the cell membrane following release into the extracellular medium and in this form are relatively resistant to proteinase inhibitors. This might be important when the close association of neutrophils with fibrin(ogen) substrate via integrin receptor interaction is considered, which leads to the formation of a "protected pocket" excluding circulating proteinase inhibitors (Weitz *et al*, 1987). The association of activated neutrophils with fibrin, with the potential for degrading these plasmin derived products, may well compromise the predictive value of circulating D-dimer laboratory markers. In addition neutrophil lysosomal enzyme proteolysis of plasmic fibrin products might account for the sometimes discrepent results in fibrinogen/FDP's and D-dimer values seen in clinical practice, where relatively lower D-dimer values than those expected from FDP levels were measured (Sato *et al*, 1995). This scenario is further complicated by the results obtained in this study by western blotting of the products produced during lysosomal enzyme digestion of the plasmic fibrin products. The reformed material is recognized by a monoclonal antibody directed against D-dimer. Wylie and Walsh (1996) reported on variable immunoreactivity of D-dimer preparations for a monoclonal antibody DD-3B6/22 and it was suggested that this may be due to extra proteolytic cleavages during fibrinolysis. A subsequent report (Rylatt and Bundesen, 1996) challenged this hypothesis as being irrelevant in the *in vivo* situation due to high systemic levels of proteinase inhibitors.

In this study we have shown that neutrophils are able to further process the end products of plasmic fibrin degradation and in doing so may have the potential for modulating the immune response and influencing the predictive value of tests measuring the levels of circulating D-dimer in certain pathological conditions. It appears that these neutrophil-derived products also have the capacity to produce high molecular weight material probably as a factor XIII and calcium dependent process. Investigation into the potential biological importance of the reformed material was beyond the scope of this study.

CHAPTER 8

Summary and conclusions

It has long been recognized that neutrophils are found within thrombi (Rulot, 1904; Opie, 1907) and their presence, due to specific cellular adhesive processes (Palabrica *et al*, 1992; Sako *et al*, 1993; Dore *et al*, 1996) significantly increases the rate of fibrin degradation. The ability of elastase and cathepsin G, proteases present within the azurophilic granules of the neutrophil, to degrade fibrin in a manner distinct from that of plasmin has previously been shown (Moroz, 1984). This study shows that proteases of the neutrophil membrane also possess fibrinolytic potential and are operative at relatively low activation states of the cell. Although non-stimulated neutrophils were able to effect clot lysis, stimulation with PMA at a dose that does not release lysosomal enzymes increased the rate of clot solubilization. Clot lysis, which was detectable within two min of incubating cells with fibrin clot, was effected by a membrane-associated serine protease that dissociates into bands with apparent molecular weights of 501 kDa, 398 kDa, 316 kDa, 245 kDa and 209 kDa on SDS-PAGE. This appears to be the same as a previously reported membrane-associated protease which is partially resistant to MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl. inhibition and degrades both CRP and fibrinogen (Shephard *et al*, 1989; Kelly *et al*, 1994). The resulting fibrin degradation was distinct from that of plasmin, neutrophil lysosomal enzymes and pure HNE, was greater when neutrophils were incorporated within the fibrin matrix and could act together with plasmin to enhance fibrinolysis. Initial plasmin attack of the fibrin clot appears to expose neutrophil sensitive sites which allows for more efficient neutrophil-mediated solubilization to occur. This may be important in the *in vivo* situation where clot stabilization has occurred due to the release of plasminogen activator inhibitors from incorporated platelets.

Members of the integrin family of adhesion receptors have been found to be of great importance for the interaction of neutrophils with fibrinogen (Wright *et al*, 1988; Altieri *et al*, 1990 and 1993; Diamond and Springer, 1993b). In the case of neutrophil membrane fibrinolytic activity, occupation of the CD11c/CD18 receptor by the relevant monoclonal antibody, significantly inhibits this process. This suggests that the protease under investigation is situated at or in close proximity to this receptor. It was shown that association of the fibrin substrate with the cell allowed for release of the enzyme into the extracellular medium. Conditioned medium from

neutrophils pre-exposed to fibrin resulted in greater subsequent fibrinolysis than the use of conditioned medium from naive cells. Members of the integrin family have been shown to play a role both in adhesion as well as signal transduction, into and out of the cell (Pavalko and LaRoche, 1993; Petty and Todd, 1996). The results obtained indicate that crosslinked fibrin may occupy the CD11c/CD18 receptor and in doing so initiate signal transduction pathways which upregulate the enzyme. This upregulation of activity appears to be due to the activation of cellular kinases, in particular PKC with the calcium/calmodulin dependent kinases and the myosin light chain kinase being of much lesser significance.

The products generated by lysis of fibrin by neutrophil membrane serine proteases show biological activity in assays of platelet function. The low molecular weight peptides were shown to significantly inhibit platelet aggregation in a concentration dependent manner. The higher molecular weight fibrin products were able to partially overcome the resistance to plasmic lysis, which occurs in the presence of platelets (Falk, 1992). In both cases activity is presumably related to interaction of these FDP's with the platelet fibrinogen receptor, GPIIb-IIIa, although specific binding studies were not performed. Neither the peptides nor the higher molecular weight material showed any significant modulation of clot retraction, a process essential for efficient localisation of clot at the site of tissue injury, wound healing and recanalization of occluded vessels. Thus, products released following neutrophil membrane fibrinolytic activity on established thrombus may act to inhibit further platelet aggregation and improve efficiency of plasmin-mediated clot lysis, without impairing the ability of platelets to bring about clot retraction.

The classically described fibrinolytic pathway mediated by plasmin results in the formation of a number of predictable end products (Pizzo *et al*, 1973) which possess a range of biological properties (Kazura *et al*, 1989; Fischer *et al*, 1991, Robson *et al*, 1993; Lorenzet *et al*, 1992). This process may be the result of endogenous enzyme activity or the infusion of exogenous plaminogen activators for therapeutic use in a variety of thromboembolic disorders. In the latter case this results in extremely high levels of circulating FDP's (Adams *et al*, 1995). This study demonstrates that serine proteases within a lysosomal enzyme preparation released from the neutrophil are able to further process these products, initially into low molecular weight material. However with time, high molecular weight material is formed. This reformation is prevented by both calcium and factor XIII inhibitors and the products are recognized by a monoclonal antibody raised against the plasmin derived FDP D-dimer. This processing by neutrophils of the end products of plasmic fibrin degradation may have the potential for modulating the immune

response. In addition it may influence the predictive value of tests measuring circulating D-dimer, currently used as a laboratory marker for disorders such as deep vein thrombosis (Declerk *et al*, 1987), pulmonary embolism (Bounameaux *et al*, 1990) and severity of peripheral arterial occlusive disease (Lassila *et al*, 1993) as well as monitoring thrombolysis in myocardial infarction (Lew *et al*, 1986).

It is hoped that these investigations are able to provide a greater understanding of the role neutrophils play in fibrinolysis and how they may modulate the inflammatory process by the release of biologically active products into the surrounding medium.

Appendix 1

Methods

Appendix 1: Methods

Reagents

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

Iodination

[^{125}I]-Na was from Amersham International, UK. Iodogen was purchased from Pierce and Warriner, Cheshire, UK.

Polyacrylamide gel electrophoresis and isoelectric focussing

A kit containing prestained molecular weight markers for use in SDS-PAGE was obtained from Amersham International, Amersham, UK. The kit includes 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). Autoradiography was performed using Kodak XAR-5 film (Eastman Kodak, Rochester, NY). Two dimensional isoelectric focussing was performed using ampholine polyacrylamide gel plates (pH range 3.5-9.3) with an isoelectric calibration kit from Pharmacia, Uppsala, Sweden.

Monoclonal antibodies, enzymes and inhibitors

Monoclonal antibodies (all mouse IgG1) to CD16 (FcRIII, low avidity Fc receptor), CD11a (LFA-1 α chain), CD11b (Mac1 α chain), CD11c (p150,95 α chain), CD18 (β chain of β_2 integrin) and CD41a (intact GPIIb-IIIa, β3 integrin) were obtained from Sanbio, Uden, Netherlands. The monoclonal antibody to fibrin degradation product, D-dimer (mouse IgG_1) was obtained from Biogenesis Ltd., Poole, UK. 4-(2-aminoethyl)-benzenesulphonyl fluoride (AEBSF, Pefabloc® -SC) (serine protease inhibitor) and bisindolylmaleimide (protein kinase C inhibitor) were obtained from Boehringer Mannheim, Germany. 1-[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine (KN-62, Ca^{2+}/calmodulin kinase inhibitor) and 1-(5-chloronaphthalene-1 sulphonyl)-1H-hexahydro-1,4-diazepine (ML-9, myosin light chain kinase inhibitor) were obtained from Calbiochem-Novabiochem, San Diego CA, USA as was pure

human neutrophil elastase. Urokinase was from Ukidan, Rome, Italy. Plasminogen was purified from human plasma according to the method of Deutsch and Mertz (1970).

Peptide purification

Sep-Pak C_{18} cartridges were obtained from Waters Chromatography division, Millipore Corporation, Milford MA, USA. HPLC (high performance liquid chromatography) grade methanol and acetonitrile was from Baxter Healthcare Corporation, Muskegon MI, USA

Electron microscopy

Spurrs resin was obtained from from Agar Scientific Ltd, Stanstead, UK.

ECL Western blotting

Immobilon-P transfer membrane was obtained from Millipore Corporation, Milford MA, USA and the ECL (enhanced chemiluminescence) detection kit from Amersham International, UK.

Preparation and iodination of fibrinogen

Fibrinogen was isolated by ammonium sulphate precipitation from plasma obtained from heparinized blood donated by healthy laboratory workers (Atencio *et al*, 1965). Contaminating plasminogen was removed using lysine immobilized on Sepharose 4B (Deutsch and Mertz, 1970). The fibrinogen preparation contained a trace amount of factor XIII which was used to catalyze crosslinking during fibrin formation. The clottability of the purified fibrinogen was 97%. Fibrinogen was iodinated using iodogen as an oxidizing agent and 0.5 μCi[^{125}I]-Na/μg fibrinogen (Fracker and Speck, 1978). The specific activity of the ^{125}I-labelled fibrinogen was 0.5 μCi/μg protein. SDS-PAGE (non-reduced) and autoradiograph analysis (Kodak XAR-5) of this ^{125}I-labelled fibrinogen (400 000 cpm/track) revealed no evidence of degradation and it maintained its 97% clottability.

Preparation of neutrophils, conditioned medium, lysosomal enzymes and membranes

Neutrophils were separated from heparinized venous blood (5 U preservative-free heparin/ml blood) by dextran sedimentation and Hypaque/Ficoll gradient centrifugation (Boyum, 1968) and resuspended in indicator free Hanks balanced salt solution (HBSS). Neutrophil viability was 97-99% as determined by exclusion of trypan blue and found to be 98-100% pure using differential counts made on Wright-Giemsa stained smears. Neutrophil conditioned medium was prepared by

incubating neutrophils in the presence or absence of PMA (10 ng/ml) at 37°C for 20 min (Shephard *et al*, 1989). With this preparation insignificant amounts of β-glucuronidase and a maximum of 12% of the total vitamin B_{12}-binding protein of the intact cells is released (Shephard *et al*, 1989; Pontremoli *et al*, 1986). Centrifugation at 400 g for 5 min at 4°C was used to remove the cells for the preparation of the cell free conditioned medium which was used within 5 min of collection (Shephard *et al*, 1989). Lysosomal enzymes were obtained by degranulation of neutrophils in response to FMLP (1 μM) in the presence of cytochalasin B (5μg/ml) (Bentwood and Henson, 1980; Shephard *et al*, 1988). Membranes were prepared by hypotonic lysis of neutrophils and sucrose density gradient ultracentrifugation (Shephard *et al*, 1989).

Preparation and degradation of ^{125}I-labelled fibrin clot

Fibrin clots were prepared by the addition of ^{125}I-labelled fibrinogen at a final concentration of 2 mg/ml HBSS and clotted by the addition of bovine thrombin (0.4 units/ml final concentration). The clots were incubated for 2 hr (37°C), to effect crosslinking, and thereafter squeezed and added to either non-stimulated or PMA (10 ng/ml)-stimulated neutrophils, conditioned medium from non-stimulated or PMA (10 ng/ml)-stimulated neutrophils or neutrophil lysosomal enzymes in a final volume of 500 μl. Neutrophils were stimulated with PMA by addition of PMA to give a final concentration of 10 ng/ml and immediately afterwards the preformed clot was added. Conditioned medium was prepared as described above. The number of neutrophils or neutrophil equivalents/500 μl used per experiment is given in the figure legends of the relevant results section. Plasmin solubilization of ^{125}I-labelled fibrin (125 μg) was executed by the addition of a preformed clot to human plasminogen (5 or 15 μg/ml final concentration) and urokinase (final concentration 50 IU/ml) in a final volume of 500 μl HBSS. Solubilization of the preformed clot by pure human neutrophil elastase was monitored by the addition of elastase (250 ng) to the preformed clot (250 μg) in a final volume of 500 μl. Incorporation of the source of neutrophil enzyme into the fibrin matrix occurred by mixing either neutrophils, neutrophil conditioned medium or lysosomal enzymes with bovine thrombin (0.4 units/ml final concentration) followed by the addition of 1.0 mg ^{125}I-labelled fibrinogen in a final volume of 500 μl. The number of neutrophils or neutrophil equivalents/500 μl incorporated into such clots is stated in the figure legends. When the effect of enzyme inhibitors and monoclonal antibodies to leukocyte adhesion molecules (see Chapter 6) on clot degradation was investigated these were preincubated (15 min,

37°C) with the neutrophil enzyme source either prior to addition of the squeezed clot or incorporation of the enzyme source into the clot.

Degradation of the resulting fibrin clots was monitored by the release of radioactivity into the supernatant using a Packard Auto-gamma counter.

The nature of the ^{125}I-labelled protein in solution on full solubilization of the fibrin clot by PMA-stimulated neutrophils, conditioned medium from PMA-stimulated neutrophils, lysosomal enzymes, pure human neutrophil elastase or plasmin, was determined by SDS-PAGE (10% non-reducing conditions) (Laemmli, 1970). For this an aliquot of the soluble protein was mixed with an equal volume of 4% SDS sample buffer and boiled. Gels were immediately dried and the products visualized by autoradiography.

The molecular size of the neutrophil fibrin-degrading protease

A neutrophil membrane preparation (Shephard *et al*, 1989) from 230x10^6 cells was separated by SDS-PAGE (3-13%) under non-reducing conditions. A kit containing pre-stained molecular weight markers for molecular weights 14-200 kD, ferritin (subunit size 220 kD), purified human fibrinogen (340 kD) and fibronectin (440 kD) were used as calibration standards. After removal of SDS (Granelli-Piperno and Reich, 1978) the gel was cut into 2 mm slices. The slices were assayed for enzyme activity by incubating each slice (37°C, 24 hr) with ^{125}I-labelled fibrin clots (250 μg) in a final volume of 250 μl. Solubilization was monitored by the release of radioactivity into the supernatant.

Degradation by neutrophil lysosomal enzymes of the end products of plasmin digestion

^{125}I-labelled fibrinogen at a final concentration of 1 mg/ml HBSS was clotted by the addition of bovine thrombin at 0.4 units/ml final concentration. The clots were incubated for 2 hr (37°C), to effect crosslinking, and thereafter squeezed and solubilized (24 hr, 37°C) by plasmin (Deutsch and Mertz, 1970) by the addition of urokinase (50 IU/ml) and human plasminogen (40 μg/ml) in a final volume of 250 μl. Neutrophil lysosomal enzyme preparation from 5x10^6 cells, pure human leukocyte cathepsin G (25 μg/mg protein) or pure human neutrophil elastase (25 μg/mg protein) was then added to the end products (250 μg) of plasmic ^{125}I-labelled fibrin degradation (final volume 1 ml). When required the reactions were done in the presence of the thiol protease inhibitors leupeptin (20 μM) and trans-epoxysuccinyl-L-leucylamido-(4-guanido) butane (E64, 400 μM), the chymotrypsin inhibitor L-1-tosylamido-2-phenyl-ethyl-chloromethylketone (TPCK,

500 μM), the trypsin inhibitor α_1-p-tosyl-L-lysine-chloromethyl-ketone (TLCK, 500 μM), the metalloprotease inhibitor ethylene diamine tetra acetic acid (EDTA, 10 mM), the serine protease inhibitors 4-(2-aminoethyl)-benzene sulfonyl fluoride (AEBSF, 1 mM) and phenylmethylsulphonyl fluoride (PMSF, 1 mM), the cathepsin G inhibitor Suc-$(Ala)_2$-Pro-PheCH_2Cl (1 mM), the neutrophil elastase inhibitors α_1-antitrypsin (1.5 mg/ml) and MeO-Suc-$(Ala)_2$-Pro$ValCH_2Cl$ (10 μM) and DL-dithiothreitol (1 mM), a reagent used for maintaining sulfhydryl groups in their reduced state. These inhibitors were preincubated (15 min, 37°C) with the neutrophil lysosomal enzyme preparation before the addition of the plasmic ^{125}I-labelled-fibrin digest. The effect of spermine and spermidine (10 mM and 100 mM), primary amines that inhibit factor XIII, on the nature of the lysosomal enzyme degraded ^{125}I-labelled fibrin degradation products was also investigated by incubating spermine and spermidine with ^{125}I-labelled fibrin clots during plasmin digestion. The nature of the ^{125}I-labelled fibrin degradation products was analysed by SDS-PAGE (10%) (Laemmli, 1970) in the absence of reducing conditions, except where indicated in the results, by boiling an aliquot of the degradation products with an equal volume of 4% SDS sample buffer. Gels were immediately dried and the products visualized by autoradiography.

Two-dimensional iso-electric focusing of plasmin-derived fibrin degradation products

^{125}I-labelled fibrin degradation products generated by plasmin at 24 hr and products generated by neutrophil lysosomal enzyme action on these plasmin-derived products were dialysed against water overnight at 4°C, freeze dried and resuspended at 4 mg/ml in 20 mM Tris (pH 8.3), 1% (w/v) sodium dodecyl sulphate and 7 M urea (Strachan *et al*, 1988). Samples were loaded onto a prefocused Ampholine polyacrylamide gel (PAG) plate (5% polyacrylamide with 3% crosslinkage and 2.2% [w/v] ampholine) with a broad pH range between 3.5 and 9.3. An isoelectric calibration kit containing standards with a pH range between 3.5 and 9.3 was included. The proteins were focused on the PAG plate with a constant power of 15 W, 1 500 V and 25 mA at 10°C then stained with Coomassie (0.12%), dried between two sheets of polyester film and autoradiographed. For second dimension SDS-PAGE (10%, non-reduced) (Laemmli, 1970) followed by autoradiography the PAG plate was sealed above the polyacrylamide gel with 1% low melting agarose in SDS-sample buffer (0.004 M Tris [pH 6.8] with 4% [w/v] SDS and 24% [v/v] glycerol).

Electrophoresis and immunoblotting of plasmin-derived fibrin degradation products

Fibrin degradation products generated by plasmin at 24 hr and products generated by neutrophil lysosomal enzyme action on these plasmin-derived products over a time period of 24 hours were subjected to SDS-PAGE (non-reduced, 10%) (Laemmli, 1970). The separated proteins were transferred to an Immobilon-P transfer membrane (Towbin *et al*, 1979), thereafter non-specific binding sites were blocked by overnight incubation (room temperature) in 5% blocking solution (50 mM tris, 80 mM NaCl, 2% BSA, 0.2% NP-40 and 5% fat-free milk powder; pH 8). All subsequent steps were performed using a 2% blocking solution. The Immobilon-P membrane was then probed using a monoclonal antibody directed against human D-dimer (a control track using no primary antibody was also set up) at 5 μg/ml final concentration. Antigen/antibody binding was made visible with appropriate antibody-peroxidase conjugates. An ECL detection kit was used in the final development (Andrews, 1986).

Preparation of fibrin degradation products (FDP's) and fibrin peptides by neutrophil conditioned medium degradation of fibrin

Both the entire FDP mix formed by neutrophil conditioned medium degradation of fibrin and the peptide component alone were used in experiments to investigate aspects of platelet function. For this neutrophil conditioned medium was incorporated within fibrin clot then incubated for 24 hr (37°C) to effect full clot solubilization. The low molecular weight fibrin peptides were prepared by the addition of 50% TCA (w/v) to the degradation mix (0°C) to a final concentration of 10%. Insoluble protein was removed by centrifugation (400g for 10 min at 4°C) and TCA was extracted from the soluble peptide mix four times with four volumes of diethyl ether (Shephard *et al*, 1988). The peptides were freeze dried and then resolubilized in a tenth of the original volume with distilled water. The peptides were desalted using a Sep-Pak C_{18} cartridge after treatment with methanol and equilibration with 0.1% trifluoroacetic acid (TFA) (4 column volumes) according to manufacturers instructions. Peptides were then loaded onto the column and after washing with 0.1 % TFA, the adsorbed peptides were eluted with 100% acetonitrile. After evaporation of the solvent, peptides were redissolved in HBSS. An FDP mix control and peptide control were produced by processing neutrophil conditioned medium in the same manner as described above, but without the presence of fibrinogen.

Electron microscopy

Fibrin samples with and without neutrophils (± PMA) incorporated within the matrix were processed for viewing by electron microscopy. At selected time points the clot was fixed in 2.5% gluteraldehyde in phosphate buffer.

Scanning electron microscopy

Following washing, the clot preparations were dehydrated through a graded series of ethanol concentrations and dried from CO_2 by the critical point method. The samples were sputter coated with gold palladium and examined in a LEICA S440 scanning electron microscope (Cambridge, UK).

Transmission electron microscopy

Following washing the clot preparations were post fixed in osmium tetroxide for 2 hr, rinsed in distilled water and dehydrated through a graded series of ethanol concentrations. Thereafter the samples were placed in 100% acetone and embedded in Spurr's resin. The embedded samples were sectioned using a Leica Ultracut S microtome (Vienna, Austria) and viewed using the JEOL 200CX transmission electron microscope (Tokyo, Japan).

Platelet purification

The method of platelet purification was a modification of that described by Mustard *et al* (1989). Blood was collected via venipuncture with a 19-gauge needle from healthy adult volunteers. These donors were on no medication known to interfere with coagulation or platelet function for at least 10 days prior to donation. Blood samples were collected in one-sixth volume CCD (93 mM trisodium citrate, 7 mM citric acid, 140 mM dextrose; pH 6.5) with 0.35% BSA at 37°C. Platetlets do not aggregate readily at this pH, while BSA is a protective protein which prevents activation during the purification process (Mustard *et al*, 1989). This blood was centrifuged at 200 g (20 min, 26°C) to obtain platelet rich plasma and thereafter made up with an equal volume of CCD with 0.35% BSA and the platelets pelleted (990 g, 10 min). The pelleted platelets were then washed in PBS (pH 7.4) with 0.35% BSA. Aprrase and PGI_2 were not included in the isolation buffers. Final resuspension was then performed with HBSS with 3.35% BSA. Platelets in this medium have been shown to be responsive to all aggregating and release-inducing agents such as thrombin, PAF and sodium arachidonate (Mustard *et al*, 1989). The purified platelets were used in a series of experiments to investigate platelet aggregation (platelet aggregometer from Chronolog Corporation, Havertown, Pa, USA), clot retraction and plasmic degradation of platelet-rich fibrin clots. Experimental details of platelet aggregation and clot retraction are described in Chapter 6.

Appendix 2: Abbreviations

ADP	adenosine diphosphate
AEBSF	(2-aminoethyl)-benzenesulphonyl fluoride
AGP7	azurophil granule protein-7
ATP	adenosine triphosphate
BSA	bovine serum albumin
C-terminus	carboxyl-terminus
cAMP	cyclic adenosine monophosphate
CCD	citrate-citric acid-dextrose buffer
CRP	C-reactive protein
DFP	diisopropyl fluorophosphate
E64	trans-epoxysuccinyl-L-leucyl-amido(4-guanidino) butane
EACA	ε-amino-N-caproic acid
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FDP	fibrin degradation product
FMLP	N-formyl-methionyl-leucyl-phenylalanine
GM-CSF	granulocyte macrophage colony stimulating factor
GTP	guanosine triphosphate
H7	1-(5-isoquinolinesulphonyl)-2-methylpiperazine
HBSS	Hanks balanced salt solution
HNE	human neutrophil elastase
HPLC	high performance liquid chromatography
IAP	integrin associated protein

Ig	immunoglobulin
ICAM	intercellular adhesion molecule
IFN	interferon
IL-6	interleukin-6
IL-8	interleukin-8
KN-62	[N,O-bis-(5-isoquinolinesulfonyl)-N-methyl-L-tyrosyl]-4-phenylpiperazine
LRI	leukocyte reponse integrin
LTB	leukotriene
MAP kinase	mitogen-activated protein kinase
ML9	hexahydro-1,4-diazepine
MMP	matrix metalloproteinase
MMP-8	neutrophil collagenase
MMP-9	neutrophil gelatinase
N-terminal	amino-terminal
NADPH	nicotinamide adenine dinucleotide phosphate
PAG plate	ampholine polyacrylamide gel plate
PAF	platelet activating factor
PAI-1	plasminogen activator inhibitor-1
PDGF	platelet derived growth factor
PF4	platelet factor-4
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMN	polymorphonuclear leukocyte
PR3	proteinase-3
PSGL-1	P-selectin glycoprotein ligand-1
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TCA	trichloroacetic acid

TFA	trifluoroacetic acid
TFP	trifluoperazine
TLCK	α_1-p-tosyl-L-lysine-chloromethyl-ketone
TNF	tumour necrosis factor
TPCK	L-1-tosylamido-2-phenyl-ethyl-chloromethylketone
tPA	tissue-type plasminogen activator
uPA	urokinase-type plasminogen activator
uPA-R	urokinase-type plasminogen activator receptor
VCAM	vascular cell adhesion molecule
vWF	von Willebrand factor

Appendix 3: Experimental data

Chapter 5

Figure 2: page 66

Solubilization of preformed ^{125}I-labelled fibrin clot by various enzyme sources. The results shown are the range of values obtained from 10 different experiments.

A. With respect to enzyme levels at 60 min. Reactions contained the indicated number of neutrophils or neutrophil equivalents and 250 μg preformed ^{125}I-labelled fibrin clot.

	^{125}I-labelled fibrin solubilized (μg) at 60 min Cell number			
	1.25 x 10^6	**2.5 x 10^6**	**5 x 10^6**	**10 x 10^6**
Conditioned medium	5 - 7	7 - 8	7 - 8	8 - 9
Conditioned medium (PMA)	9 - 10	12 - 14	14 - 16	20 - 22
Neutrophils	14 - 18	26 - 29	30 - 34	29 - 32
Neutrophils (PMA)	19 - 21	31 - 38	34 - 38	40 - 44
Lysosomal enzyme	35 - 39	39 - 43	55 - 61	81 - 87

B. With respect to clot size at 60 min. Reactions contained 2.5x10^6 neutrophils or neutrophil equivalents and the indicated weight of preformed ^{125}I-labelled fibrin clot.

	^{125}I-labelled fibrin solubilized (μg) by 2.5 x 10^6 neutrophils or neutrophil equivalents at 60 min		
	125 μg clot	**250 μg clot**	**1000 μg clot**
Conditioned medium (PMA)	5 - 6	12 - 14	64 - 72
Neutrophils (PMA)	15 - 17	33 - 37	106 - 114
Lysosomal enzyme	24 - 27	40 - 44	176 - 193

C. With respect to time. Reactions contained 125 μg preformed ^{125}I-labelled fibrin and either 2.5x 10^6 neutrophils or neutrophil equivalents or plasmin generated by activation of 5 μg or 15 μg plasminogen/ml with 0.4 IU urokinase/ml.

	^{125}I-labelled fibrin solubilized (μg) by2.5 x 10^6 neutrophils or neutrophil equivalents or 5/15 μg plasminogen		
	30 min	**60 min**	**90 min**
Conditioned medium	2 - 3	7 - 8	11 - 12
Neutrophils	7 - 8	16 - 18	21 - 23
Lysosomal enzyme	11 - 13	24 - 27	27 - 30
Plasminogen (5 μg)	1 - 3	3 - 6	31 - 37
Plasminogen (15 μg)	4 - 6	16 - 18	65 - 73

Figure 3: page 68

Comparison of clot solubilization by neutrophils added to a preformed 1 mg ^{125}I-labelled fibrin clot and neutrophils incorporated into a 1 mg ^{125}I-labelled fibrin matrix. Reactions contained either 2.5x10^6 neutrophils or neutrophil equivalents. The results shown are the range of values obtained in 10 different experiments.

	^{125}I-labelled fibrin solubilized (μg) from 1000 μg clot	
	Addition of enzyme source to preformed clot	**Incorporation of enzyme source into clot**
Conditioned medium	30 - 34	143 - 154
Conditioned medium (PMA)	48 - 51	269 - 311
Neutrophils	111 - 120	361 - 373
Neutrophils (PMA)	128 - 139	422 - 450
Lysosomal enzyme	213 - 222	687 - 731

Figure 4: page 69

Influence of PMA-stimulated neutrophils ($2.5x10^6$) or PMA-stimulated neutrophil conditioned medium (from $2.5x10^6$ neutrophils) on plasmin solubilization of a 125 μg preformed ^{125}I-labelled fibrin clot. Each data point is the mean value of triplicates (which did not differ by more than 2%). The results from 3 replicate experiments is given. *Result illustrated in figure 4.

	^{125}I-labelled fibrin solubilized (μg)								
	***Experiment 1**			**Experiment 2**			**Experiment 3**		
	30	**60 min**	**90**	**30**	**60 min**	**90**	**30**	**60 min**	**90**
Conditioned medium (PMA)	8	16	24	6	15	22	7	16	25
Neutrophils (PMA)	4	7	12	4	6	11	3	7	13
Conditioned medium (PMA) + plasmin at "0"	34	66	100	31	68	106	29	69	103
Neutrophils (PMA) + plasmin at "0"	22	31	77	20	30	75	23	32	77
Neutrophils (PMA) + plasmin at 30 min	5	40	91	6	38	87	7	39	92
Neutrophils (PMA) + plasmin solubilization for 30 min then addition of EACA + aprotinin	2	30	60	2	28	57	1	32	59

Insert Table 1: page 72

Solubilization (60 min) of preformed ^{125}I-labelled fibrin (250μg) clot by 2.5 x 10^6 PMA-stimulated neutrophils or lysosomal enzymes from 2.5 x 10^6 neutrophils or 250 ng pure human neutrophil elastase in the presence of enzyme inhibitors as indicated. The results shown are the range of values for 4 replicate experiments.

Inhibitor	^{125}I-labelled fibrin solubilized (μg)
PMA-stimulated neutrophils	
None	34 - 38
EACA (25 mM)	37 - 40
Aprotinin (20 units/ml)	36 - 38
EDTA (5 mM)	37 - 40
E64 (20 mM)	36 - 38
Suc-$(Ala)_2$-Pro-PheCH_2Cl (1mM)	38
Z-Gly-Leu-PheCH_2Cl (0.1 mM)	35 - 37
Suc $(Ala)_2$-Val-CH_2Cl (1 mM)	37
Suc-$(Ala)_3CH_2Cl$ (1 mM)	37
AEBSF (1 mM)	1 - 4
MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM)	22 - 24
Neutrophil lysosomal enzymes	
None	48 - 50
Z-Gly-Leu-PheCH_2Cl (0.1 mM)	26 - 29
AEBSF (1 mM)	2 - 6
MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM)	7 - 11
Pure human neutrophil elastase	
None	67 - 71
AEBSF (1 mM)	1 - 4
MeO-Suc-$(Ala)_2$-Pro-ValCH_2Cl (0.1 mM)	1 - 4

Table 3: page 77

Influence of monoclonal antibodies directed against various neutrophil receptors on ^{125}I-labelled fibrin degradation (60 min) by PMA-stimulated neutrophils ($2.5x10^6$ neutrophils/1 mg fibrin clot) incorporated into fibrin matrix. The results shown are the mean values obtained of triplicates (that did not vary by more than 5%) in 2 separate experiments. The cost of antibodies did not permit the performance of more experiments.

*Results illustrated in Table 3.

Monoclonal antibody	% Inhibition of degradation	
	Experiment 1*	Experiment 2
None	0	0
anti-CD 11a	15	14
anti-CD 11b	13	12
anti-CD 11c	58	60
anti-CD 18	58	59
anti-CD 16	0	1
anti-CD 41	0	0

Table 4: page 78

Influence of various kinase inhibitors on fibrin degradation by non-stimulated and PMA-stimulated neutrophils at 60 min ($2.5x10^6$ neutrophils/1 mg fibrin clot). The results shown are the range of values obtained in 3 experiments.

Inhibitor	% Inhibition (-PMA)	% Inhibition (+PMA)
None	0	0
ML-9	0 - 2	12 - 14
Bisindolylmaleimide	10 - 12	32 - 36
KN-62	2 - 4	13 - 21

Chapter 6

Table 1: page 86

Plasmic degradation of ^{125}I-labelled fibrin clot at 60 min with modification by incorporated platelets and incorporated platelets together with PMA-stimulated neutrophils ($2.5x10^6$ neutrophils/$2x10^8$ platelets/125 μg ^{125}I-labelled fibrin clot). Illustrated are the range of results obtained from 3 separate experiments.

Constituents of clot	**^{125}I-labelled fibrin released (μg)**	**% Inhibition of plasmin degradation**
UK / plg	97 - 99	-
UK / plg + plt	48 - 50	49 - 51
UK / plg + plt + neutrophils	71 - 73	26 - 28

Table 2: page 90

Plasmic degradation of ^{125}I-labelled fibrin clot (250 μg) at 60 min and modification by incorporated platelets, alone or preincubated with FDP, FDP control, a peptide control or peptides derived from neutrophil conditioned medium solubilization of fibrin clot (1.5 mg FDP or peptides/$2x10^8$ platelets/250 μg ^{125}I-labelled fibrin clot). Illustrated are the range of results obtained from four replicate experiments.

Constituents of clot	**^{125}I-labelled fibrin released (μg)**	**% Inhibition of plasmin degradation**
UK / plg	181 - 185	0
UK / plg + plt	56 - 64	65 - 70
UK / plg + plt + FDP	98 - 116	37 - 47
UK / plg + plt + FDP control	61 - 69	63 - 67
UK / plg + plt + peptides	53 - 59	68 - 71
UK / plg + plt + peptide controls	51 - 59	68 - 72

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