Angiopoietin-2 and Platelet-Derived Growth-BB

Factor Cooperatively Affect Peripheral

Blood Monocyte Fibrinolysis

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Abstract

In almost all instances of adult angiogenesis, the growth of new vessels is associated with inflammation. An inflammatory infiltrate composed mainly of monocytes and neutrophils precedes or accompanies angiogenesis. Once inside the wound environment, monocytes migrate through the temporary scaffold provided by the fibrin clot, and differentiate into macrophages. In this study the interaction between peripheral blood monocytes and two angiogenic growth factors was investigated.

Angiopoietin-2 (Ang-2) is a growth factor originally identified as playing a critical role in vessel remodeling during angiogenesis. It was recently shown to be a component of the endothelial cell Weibel-Palade bodies, suggesting involvement in vascular homeostatic responses such as coagulation and inflammation, which are central to wound healing. Platelet-derived growth factor-BB (PDGF-BB) is a member of the pleiotrophic family of PDGFs, and is a known component of the α-granules of platelets. Both these growth factors are therefore temporally and spatially co-expressed during the formation of the fibrin clot, and the arrival of the monocyte infiltrate.

In this study we show that the co-administration of Ang-2 and PDGF-BB to peripheral blood monocytes in vitro causes a significant upregulation in monocyte fibrin clot invasion through fibrinolysis. This heightened invasion was dependent on both serine proteases and matrix metalloproteinases (MMPs), and the combination of Ang-2 and PDGF-BB upregulated urokinase plasminogen-activator receptor (uPAR) expression, as well as MMP9 and membrane type 1 MMP (MT1-MMP).

No expression of the Ang-2 receptor, Tie-2, was seen on the peripheral blood monocytes under experimental conditions. Instead, Ang-2 was seen to signal to the cells through the leukocyte-specific integrin β2. Furthermore, the occupancy of integrin β2 with Ang-2 was seen to increase the integrin's association with uPAR, while the inhibition of integrin β2 removed the Ang-2/PDGF-BB-dependent upregulation of MMP9 and MT1-MMP.

PDGFR-β was poorly expressed on untreated monocytes, however treatment with Ang-2 (with or without PDGF-BB) significantly raised the expression of the receptor. This suggests that one of the ways in which Ang-2 and PDGF-BB interact is through the Ang-2 dependent facilitation of PDGF-BB/PDGFR-β signaling.
The Ang-2/PDGF-BB-dependent upregulation of fibrin invasion was seen to extend to monocytes from different species, as determined by rat and baboon peripheral blood monocytes. This fibrin invasion, however, did not extend to other cell types, such as endothelial cells, smooth muscle cells and fibroblasts. This suggested that the association of Ang-2 to integrin $\beta_2$ is critical for the Ang-2/PDGF-BB action.

Proteolytic events at the cell surface, such as cell migration, are of interest because of their potential to affect cellular functions immediately (Seiki 2002), and the finding of elements of the angiogenic cascade affecting monocyte fibrinolytic functioning is of importance. In this project the interaction between monocytes and members of the angiogenic cascade are studied in terms fibrinolytic potential. These data give further credence to the concept of Ang-2 as a key regulator of several essential phases of wound healing, as well as emphasizing the role of PDGF-BB in the process. It also provides an initial analysis of the manner in which Ang-2 and PDGF-BB act in a cooperative manner to signal to peripheral blood monocytes.
Thanks

First and foremost, I would like to thank my supervisor, Dr Neil Davies, for his continued guidance, patience, and humour. His impeccable insight and daunting knowledge has made this entire thesis an exciting and unforgettable experience!

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<thead>
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<th>Full Form</th>
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<tbody>
<tr>
<td>AAV</td>
<td>Adeno-associated virus</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiopoietin</td>
</tr>
<tr>
<td>bFGF</td>
<td>Basic fibroblast growth factor</td>
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<tr>
<td>BP</td>
<td>Bullous Pemphigoig</td>
</tr>
<tr>
<td>BrdU</td>
<td>5-bromo-2'-deoxyuridine</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>COX</td>
<td>Cyclooxygenase</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FAK</td>
<td>Focal adhesion kinase</td>
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<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
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<tr>
<td>FDP</td>
<td>Fibrin degradation product</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GFR</td>
<td>Growth factor reduced</td>
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<tr>
<td>hPU</td>
<td>Heparin-coated polyurethane</td>
</tr>
<tr>
<td>HBEGF</td>
<td>Heparin-binding EGF-like growth factor</td>
</tr>
<tr>
<td>HBS</td>
<td>Heps buffered saline</td>
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<tr>
<td>HSVEC</td>
<td>Human saphenous vein endothelial cell</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical endothelial cell</td>
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<tr>
<td>ICAM</td>
<td>Intercellular adhesion molecule</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
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<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
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<td>MCP</td>
<td>Monocyte chemotactic protein</td>
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<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>Membrane-type MMP</td>
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<td>MVEC</td>
<td>Microvascular endothelial cells</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<td>PAI</td>
<td>Plasminogen activator inhibitor</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>Platelet-derived growth factor receptor</td>
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<tr>
<td>Pe</td>
<td>Phycoerythrin</td>
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<td>Phosphoinositide 3-kinase</td>
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<td>Protein kinase-C</td>
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<td>Phorbol-12 myristate 13-acetate</td>
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<tr>
<td>PMC</td>
<td>Platelet-monocyte complexes</td>
</tr>
<tr>
<td>PSGL</td>
<td>P-selectin glycoprotein ligand</td>
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<tr>
<td>rpm</td>
<td>Revolutions per minute</td>
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<td>Reverse transcription polymerase chain reaction</td>
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<td>SCM</td>
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<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
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<td>SFM</td>
<td>Serum-free medium</td>
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<td>Smooth muscle cell</td>
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<tr>
<td>TF</td>
<td>Tissue factor</td>
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<tr>
<td>TGF</td>
<td>Transforming growth factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>Tissue inhibitor of metalloproteinases</td>
</tr>
<tr>
<td>t-PA</td>
<td>Tissue-type plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>Urokinase-type plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>Urokinase-type plasminogen activator receptor (CD87)</td>
</tr>
<tr>
<td>VCAM</td>
<td>Vascular cell adhesion molecule</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vWF</td>
<td>Von Willebrand's factor</td>
</tr>
</tbody>
</table>
1

Introduction

1.1 Angiogenesis and Inflammation

1.1.1 Angiogenesis and Wound Healing

Angiogenesis is a key process in wound healing (Carmeliet 2000), during which blood vessels are formed through a highly controlled sequential process (Davis 2002; Mariotti 2006). The angiogenic process is also responsible for the successive remodeling of blood vessels formed during embryonic vasculogenesis, pruning vessels into the highly developed adult vascular system, and in the maintenance of the female reproductive cycle (Carmeliet 2000; Conway 2001; Kampfer 2001). As angiogenesis plays a significant role in many pathological diseases, such as cancer, understanding and controlling the angiogenic process remains an area of intensive research (Carmeliet 2000).

Initiation of angiogenesis scenarios is accompanied by hyper-permeability of the vasculature (Dvorak 1995), which leads to plasma exudation and deposition of plasma proteins (Hiraoka 1998). Fibrinogen, a major component of plasma, polymerises in the presence of tissue factor (TF) and/or platelet degranulation, leading to formation of a fibrin clot (Hiraoka 1998). This establishment of haemostasis by the fibrin clot is vital for the progression of healing, and the clot is believed to further serve as a temporary matrix which supports the migration of cells involved in the formation of a new vessel sprout (Dvorak 1995; van Hinsbergh 2001).

The angiogenic process is controlled by a number of different angiogenic and anti-angiogenic factors, and recent studies have already identified a number of key players in the angiogenic cycle, such as vascular endothelial growth factor (VEGF) – a critical driver in vessel remodeling, and mediator of initial vessel hyper-permeability (Davis 2002; Mariotti 2006). The complex array of factors necessary for effective angiogenesis are tightly regulated (Conway 2001), and are produced by a variety of different sources. While a number of angiogenic signals are produced by endothelial cells, many others come from cells in nearby tissues, such as keratinocytes, corneal fibroblasts, synovial fibroblasts, monocytes and macrophages (Jackson 1997). In addition, as cells migrate out of existing vessels, they degrade the extracellular matrix (ECM), resulting in the liberation of a plethora of growth
factors normally sequestered in the matrix (Conway 2001). Some of the major classes of molecules involved in angiogenesis are detailed in the table below.

<table>
<thead>
<tr>
<th>Class of Molecule Involved</th>
<th>Examples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth Factors and Growth factor receptors</td>
<td>Vascular endothelial growth factor (VEGF) A, B, C, D and E and VEGF receptor (R) 1, 2 and 3</td>
</tr>
<tr>
<td></td>
<td>Fibroblast growth factors (FGFs) and FGFRs</td>
</tr>
<tr>
<td></td>
<td>Platelet derived growth factors (PDGFs) and PDGFRs</td>
</tr>
<tr>
<td>Adhesion molecules of the integrin and cadherin families</td>
<td>αβ1, αβ5, and VE-cadherin</td>
</tr>
<tr>
<td>Extracellular matrix (ECM) proteins</td>
<td>Fibronectin, collagens and laminins</td>
</tr>
<tr>
<td></td>
<td>von Willebrand's factor (vWF)</td>
</tr>
<tr>
<td>Remodeling and morphogenic molecules and their receptors</td>
<td>Angiopoietins and Tie receptors, Eph/ephrins</td>
</tr>
<tr>
<td>Proteases</td>
<td>MMP2, MMP9, plasminogen activators (uPA and tPA) and the inhibitors TIMPs and PAIs</td>
</tr>
<tr>
<td>Intracellular signaling molecules</td>
<td>Protein kinases (Raf and mitogen activated protein kinase (MAPK)), proteinase kinase A, B, and GTPases such as Rac and Rho</td>
</tr>
<tr>
<td>Transcription factors and regulators</td>
<td>Hypoxia inducible factor (HIF)-1-α, inhibitors of differentiation (Id1/3), nuclear factor (NF)-κB and homeobox gene products (HoxD3 and B3)</td>
</tr>
</tbody>
</table>

**Table 1. Summary of major classes of molecules involved in angiogenesis** (adapted from Ruegg 2004).

In a system such as angiogenesis, where many factors contribute to each stage of the processes, VEGF (see table 1) maintains its position as the most potent of the growth factors involved in vascular formation, and is vital both in vasculogenesis and angiogenesis (Mariotti 2006). The angiopoietins are thought to be among VEGF's most important partners, controlling the stability of the vasculature. Other growth factors, including members of the fibroblast growth factor (FGF), the platelet derived growth factor (PDGF) and the transforming growth factor β (TGF β) families also have important roles in modulating a number of different processes (Davis 2002; Mariotti 2006).

Membrane-bound factors (see table 1) also play an important in angiogenesis, and integrins, cadherins and ephrins have all been shown to be involved in vessel regeneration. Integrins, in particular, as cell adhesion molecules, have emerged as critical mediators and regulators of angiogenesis. Through physical interaction with the ECM they modulate cell adhesion,
migration and positioning, and the induction of signaling events essential for cell survival, proliferation and differentiation (Mariotti 2006).

The factors and compounds detailed in Table 1 differ not only in cell specificity, but also in the mechanisms by which they induce the growth of new blood vessels. Furthermore, many of the factors are pleiotropic, and are involved in a number of different processes specific to certain cell types. The recent studies of angiogenic factors increasingly indicate that the simplistic model of an invading capillary sprout is not sufficient to appreciate the whole spectrum of morphogenic events that are required to form a neovascular network (Augustin 2001). Many molecules that orchestrate angiogenesis control a number of functionally and molecularly related processes which remain poorly understood, such as vessel assembly, vessel maturation and extracellular matrix assembly and acquisition of vessel identity (Augustin 2001). Thus, it is becoming increasingly apparent that the angiogenic processes rely on both temporal and spatial coordination of the involved molecules in order to ensure successful vessel regeneration.

1.1.2 Inflammation and Angiogenesis

A recent study by Martin et al showed that tissue repair in immune-compromised mice (PU.1 null) was not dependent on inflammatory cells (Martin 2003). These data are some of the first that challenges the proposal of angiogenesis and chronic inflammation as co-dependent processes, involving both augmentation of cellular infiltration and proliferation, and overlapping roles of regulatory growth factors and cytokines (Jackson 1997).

While a question-mark hangs over the nature of the communication between the two processes, that damage to neonatal and adult tissues always incites an influx of inflammatory cells cannot be avoided (Jackson 1997; Martin 2003; Lemieux 2005). Indeed, post-natal angiogenesis has previously been associated with numerous inflammatory conditions, such as atherosclerosis, arthritis, retinopathy and tumour growth (Lemieux 2005), while during tissue repair, pathological remodeling or tumoral growth, an inflammatory infiltrate, composed mainly of neutrophils and monocytes precedes or accompanies angiogenesis (Jackson 1997; Croll 2004; Matias-Roman 2005; Moldovan 2005). These cells are recruited from the circulating blood in response to molecular changes in the surface of endothelial cells lining the capillaries at the wound site (Martin 1997). They migrate into the hypoxic area, and elaborate cytokines, growth factors and proteolytic
enzymes which together contribute towards the regulation of angiogenesis (Loskutoff 2000; Lemieux 2005).

Therefore, research into the role of inflammatory cells in angiogenesis – be they vital or not – continues. The complexity of the possible involvement of immune and inflammatory cells in angiogenesis has led to research in number of different areas of influence. These include: i) influence between leukocytes and endothelial cells, ii) angiogenic molecule modulation of leukocyte functions, and iii) chemokine recruitment of leukocytes and stimulation of endothelial cells. As leukocytes are also known to generate pro- and anti-angiogenic factors, their contribution towards angiogenesis, as dependent upon their temporal and spatial expression of these modulators, is also under investigation (Mariotti 2006).

1.1.3 Haemostasis and Tissue Repair

Tissue repair is initiated by the re-establishment of haemostasis, the physiologic process whereby bleeding is halted (Martin 1997). Haemostasis begins with vasoconstriction, which minimizes vessel diameter and slows bleeding. During primary haemostasis platelets bind to collagen in the exposed walls of the damaged blood vessel to form a haemostatic plug within seconds after injury. Coagulation forms the second phase of haemostasis, and involves cellular and serine protease mechanisms. It is commonly divided into two different pathways: the intrinsic and extrinsic pathways. Both constitute of a series of zymogen activations which ultimately result in the activation of thrombin. This enzyme is known to convert fibrinogen into fibrin while also activating factor XIII, which crosslinks the fibrin into the fibrin clot at the site of injury.

Secondary haemostasis also involves the degranulation of platelets (Gawaz 2005). In addition to establishing haemostasis, degranulating platelets also release a number of potent inflammatory and mitogenic substances into the local microenvironment (Martin 1997; Gawaz 2005), including PDGF-BB, TGF-β, and VEGF (Siegahn 1990; Erntoftson 1996). These growth factors attract monocytes, endothelial cells and fibroblasts (Epstein 1999; Gawaz 2005), all of which utilize the fibrin clot as a provisional matrix for their migration into the wound (Epstein 1999).

Platelet aggregation and degranulation are accompanied by endothelial activation (Matsushita 2004), a two stage process involving the rapid exocytosis of pre-formed Weibel-Palade bodies through activation by thrombin, histamine, and other agonists; and the
subsequent slower synthesis and expression of adhesion molecules (Matsushita 2004). A number of different molecules involved in rapid vascular responses related to haemostasis and inflammation have been identified in Weibel-Palade bodies. These include von Willebrand's factor (vWF), P-selectin, CD36, tissue-type plasminogen activator (tPA), and interleukin 8 (Fiedler 2004; Matsushita 2004). Interestingly, Ang-2 has also recently been identified as a Weibel-Palade body molecule (discussed later in 1.4.2.2) (Fiedler 2004; Fiedler 2006).

Exocytosis of Weibel-Palade bodies causes rapid translocation of P-selectin from within granules to the endothelial surface, where P-selectin then interacts with P-selectin glycoprotein ligand-1 on the surface of leukocytes, triggering leukocyte rolling, the first step in leukocyte trafficking (Fiedler 2004). vWF, another Weibel-Palade protein, mediates platelet rolling along the endothelium, and binds to a number of cells and molecules involved in the coagulation cascade. Thus exocytosis of Weibel-Palade bodies is a critical early step in vascular inflammation and thrombosis (Matsushita 2004).

Platelets are increasingly seen to represent an important linkage between inflammation, thrombosis and angiogenesis (Gawaz 2005), and indeed, have been shown to affect, and be affected by, all three processes. For example, platelets contribute to angiogenesis through the secretion of angiogenic factors on activation, yet the stimulation of endothelial cells with VEGF promotes adhesion and activation of platelets (Verheul 2000). Furthermore, the coagulation factor, thrombin, is required for platelet adhesion, but is also known to affect cytokine production or inflammatory cell apoptosis (Esmon 2001; Levi 2004). Other studies have demonstrated a variety of mechanisms by which coagulation, particularly the generation of thrombin, factor Xa, and the tissue factor-factor VIIa complex, can augment acute inflammatory responses, many of which are due to the activation of one or more of the protease activated receptors, and that anti-coagulants can limit the inflammatory response in wound healing situation (Esmon 2001; Levi 2004).

Clot formation and platelet degranulation during hemostasis are followed by the reorganization of intravascular clots by several cellular processes including the covering of the surface exposed to blood with monocytes, and the penetration of these cells into the clot (Moldovan 2003). Over following days, inflammatory cells, and then fibroblasts and capillaries invade the clot to form contractile granulation tissue that draws the wound's margins together. Within the granulation tissue, angiogenesis is potentiated by hypoxia,
nitric oxide, VEGF and bFGF (Epstein 1999), revascularizing and regenerating the wound area.

1.2 **Fibrinolysis and Cell Invasive Behaviour**

During wound healing the fibrin matrix serves as a structural support for neovascularization. Its highly cross-linked structure, however, presents a major barrier to cell movement (Hiraoka 1998), and fibrinolysis is essential for ingressing cells to be able to penetrate the deposited fibrin. In addition to wound healing scenarios, the fibrinolytic system also plays a vital role in the removal of fibrin from the circulation, as well as in several other biological processes including ovulation, embryogenesis, intima proliferation, angiogenesis, tumorigenesis, and atherosclerosis (Lijnen 2002).

Fibrinolysis occurs through two major groups of proteases: serine proteases and matrix metalloproteinases (MMPs) (Lijnen 2002). While the serine protease plasminogen/plasmin pathway is generally assumed to be the dominant pathway, increasing evidence suggests that MMPs may play a more important role in fibrinolysis than previously anticipated (Ronfard 2001). Indeed, it is possible that MMP- versus plasmin-dependent fibrinolytic processes may be dictated by the repertoire of proteases available to the invading cell population (Hiraoka 1998).

1.2.1 **Fibrinolysis**

![Figure 1. Schematic representation of plasminogen/plasmin and MMP fibrinolytic systems.](image)

In the plasminogen/plasmin system the proenzyme, plasminogen, is converted to the active plasmin by tPA and uPA. Plasmin degrades fibrin and also converts many pro-MMPs into active MMPs. These in turn degrade the ECM and fibrin. Activation is also regulated by positive feedback in which MMPs can activate pro-MMPs. Solid lines = activation, dotted lines = inhibition. Adapted from (Lijnen 2002).
1.2.1.1 The Plasminogen/Plasmin Cascade

The main components of the plasminogen/plasmin cascade are detailed in the schematic diagram above (figure 1). It comprises of several serine proteases: a proenzyme, plasminogen, which is proteolytically converted to the active enzyme plasmin via the action of t-PA and urokinase-type plasminogen activator (uPA) (Lijnen 2002; van Hinsbergh 2006). Several inhibitors of the serpin superfamily (PAI-1, PAI-2, α2-AP) regulate activation of plasminogen, activity of plasmin, and stepwise degradation of fibrin and other substrates (Dobrovolsky 2002). The fibrinolytic activity of the plasminogen/plasmin cascade depends on the activity of the enzyme plasmin. In addition to fibrin, however, this protease is known to have wide specificity and directly cleaves blood coagulation factors V/Va and VIII/VIIIa, some growth factors, MMPs and other extracellular matrix components. It can also catalyze the degradation of plasmin-resistant matrix proteins, such as natural collagens, through activation of inactive zymogens of collagenases (Ellis 1997; van Hinsbergh 2006). Thus, due to its multifarious substrates, the plasminogen/plasmin cascade is thought to play a dominant role in extracellular proteolysis throughout the body (Parfyonova 2002).

The precise timing and location of adequate extracellular proteolysis is provided by multiple mechanisms, among which the regulation of expression of plasminogen activators and inhibitors is especially important. A wide spectrum of hormones, growth factors, and cell environment factors are involved in the regulation of expression of the genes of plasminogen activators and of their inhibitors. The expression of uPA, for example, is activated by various inflammatory factors such as cytokines (Besser 1996), growth factors (Reid 1999), and tumour promoters (Stoppelli 1986). Conversely, anti-inflammatory agents, such as glucocorticoids have been seen to inhibit its expression (Parfyonova 2002). The regulation usually occurs on the level of gene transcription, but can also occur on the level of translation and protein secretion. Once secreted by the cells, members of the cascade are able to enter circulation. Table 2 demonstrates the components of the plasminogen/plasmin system, and lists their function, half-life and the mean plasma concentration.
<table>
<thead>
<tr>
<th>Component</th>
<th>Molecular mass kDa</th>
<th>Mean plasma conc</th>
<th>Half-life</th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasminogen</td>
<td>90</td>
<td>0.2 mg/ml</td>
<td>50 hours</td>
<td>Pro-enzyme</td>
</tr>
<tr>
<td>tPA</td>
<td>70</td>
<td>5 - 10 ng/ml</td>
<td>2 - 3 min</td>
<td>Plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>55, 31</td>
<td>1 ng/ml</td>
<td>3 - 5 min</td>
<td>Plasminogen activator</td>
</tr>
<tr>
<td>PAI-1</td>
<td>50</td>
<td>60 ng/ml</td>
<td>5 - 7 min</td>
<td>Inhibitor of tPA and uPA</td>
</tr>
<tr>
<td>PAI-2</td>
<td>70</td>
<td>&lt; 5 ng/ml</td>
<td>5 - 7 min</td>
<td>Inhibitor of uPA and tPA</td>
</tr>
<tr>
<td>α2-antiplasmin</td>
<td>70</td>
<td>0.07 mg/ml</td>
<td>50 hours</td>
<td>Plasmin inhibitor</td>
</tr>
</tbody>
</table>

Table 2. Components of the plasminogen/plasmin fibrinolytic system. Adapted from (Collen 2001; Dobrovolsky 2002).

Although the activators tPA and uPA are products of two different genes, they have in common the same substrate, plasminogen, and two specific inhibitors: plasminogen activator inhibitors 1 and 2 (PAI-1 and -2) (Parfyonova 2002). In their manner of expression, however, they have been seen to differ considerably, in that tPA is expressed in relatively few tissues or cell types, its primary source probably being the endothelium, while uPA is expressed by many cell types in a variety of tissues (Ellis 1997). tPA usually functions as a secreted enzyme, and is involved in general fibrinolysis needed to maintain vessel patency (Parfyonova 2002). Although there have been reports of tPA binding to the cell surface of SMCs (Grobmyer 1993; Ellis 1997), uPA is usually considered to be the primary plasminogen activator involved in pericellular proteolysis due to its binding to the cell surface by a membrane-bound uPA receptor (uPAR) and the generation of cell-bound plasminogen/plasmin (Ellis 1997; Lijnen 2002; Ellis, 1997 #674).

uPAR is a GPI-receptor, and lacks both cytoplasmic and transmembrane domains. This allows it a high degree of lateral mobility in the cell membrane - its location and therefore that of the urokinase bound to it are very mobile and closely associated with the functional state of the cell. Thus, in migrating cells, the uPAR is seen to cluster on the leading edge, focusing the proteolytic potential on the cell-substrate and cell-cell contact sites necessary for directed cell movement (Parfyonova 2002). uPA-uPAR is therefore thought to be mainly involved in cell migration (Collen 2001), suggesting that it is a key factor in physiological and pathological processes in which matrix degradation and tissue remodeling are required (Padro 2002).
In invading leukocytes, for example, a complex containing uPA, uPAR, and integrins has been recognized as vital for cell adhesion and invasion (Simon 1996; Pluskota 2003; Tarui 2003; Pluskota 2004; van Hinsbergh 2006), and in vivo, uPAR-deficient mice show significantly reduced leukocyte recruitment to inflamed areas (Prager 2004). An increase in functional uPA-uPAR has been detected at the cell surface of the leading edge of migrating leukocytes, allowing the substrate, plasminogen to be cell-associated, and focused on the leading edge of the migrating cell (Murphy 1999). The repetitive activation of the uPA/plasmin system at adhesion sites enables migrating leukocytes to tunnel through fibrinous matrices. This interaction can be on the cell surface, but also between uPAR and integrins on another cell, suggesting that the uPAR-integrin interaction facilitates cell-matrix and even cell-cell interactions (Tarui 2003; van Hinsbergh 2006). Indeed, uPAR's ability to interact with proteins other than uPA has lead to the discovery of its role as a multifunctional receptor. uPAR has been observed to be involved in several different, yet often related biological events in addition to cell-associated proteolysis, such as cell adhesion, chemotaxis, cell migration, and proliferation (Montuori 2002).

While the plasminogen/plasmin system is known to be an important fibrinolytic mechanism, the recent discoveries of the multifunctional nature of some of the elements of this system, such as plasmin and uPAR, suggest that the activation of the plasminogen/plasmin system cannot be seen simplistically in terms of fibrinolysis. In addition, fibrin-rich tissues in plasminogen-null animals are infiltrated efficiently by normal as well as neoplastic cell types despite significant defects in intravascular fibrinolysis (Hotary 2002). These observations suggest the presence of alternate proteolytic systems that would allow cells to infiltrate fibrin matrices via plasminogen-independent processes (Hotary 2002).

1.2.1.2 Matrix Metalloproteinases

MMPs are a family of over 20 zinc-dependent endopeptidases involved in matrix degradation and remodeling (Johnson 1998; McCawley 2001). MMPs have a wide range of substrates, including matrix components such as cross-linked fibrin, collagen, gelatin, and elastin (Johnson 1998). They have been shown to regulate developmental growth as well as many remodeling processes, such as angiogenesis, arterial remodeling and fetal development, cell migration and invasion, and wound healing (Johnson 1998; McCawley 2001; van Hinsbergh 2006). MMPs also play an important role in the cleavage and activation of various growth factors (McCawley 2001). Due to their highly influential role in tissue remodeling, MMP
functioning is controlled by a delicate balance between the MMPs and natural tissue inhibitors of metalloproteases (TIMPs) and other MMP inhibitors (Chang 2001; Baker 2002).

All MMPs are synthesized as inactive zymogens (Van 1990) and activated in situ by enzymatic means. This activation occurs through the enzymatic breakage of the "cysteine switch", which opens the active site (Van 1990; Worley 2003; van Hinsbergh 2006). MMPs can be categorized depending on their position as active enzymes and can be classed as either soluble or membrane type MMPs (MT-MMP). While all soluble MMPs are secreted in the latent form and activated in the extracellular environment, MT-MMPs are activated intracellularly by furin-like enzymes and anchored to the plasma membrane (Cowell 1998; Lafleur 2001; Murphy 2002; van Hinsbergh 2006).

All MMPs demonstrate very low activity in normal tissue (Shapiro 1998; van Hinsbergh 2006), but are upregulated and/or activated during inflammation and physiological remodeling processes in response to specific stimuli, including cytokines, growth factors and extracellular matrix interactions (Murphy 1995; Johnson 1998). In these situations, MMPs are synthesized by endogenous connective tissue cells, as well as some types of haematopoietic cells (Murphy 1995). Indeed, recent investigations have demonstrated that the extracellular matrix can directly influence its remodeling and repair via regulation of the production of MMPs by resident inflammatory cells through enzyme- and substrate-specific responses (Shapiro 1993).
<table>
<thead>
<tr>
<th>Name</th>
<th>Alternative Name</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Collagenases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP1</td>
<td>Interstitial collagenase</td>
<td>Pro-MMP2, Pro-MMP9, helical collagen</td>
</tr>
<tr>
<td>MMP8</td>
<td>Neutrophil collagenase</td>
<td>Helical collagen</td>
</tr>
<tr>
<td>MMP13</td>
<td>Collagenase 3</td>
<td>Helical collagen</td>
</tr>
<tr>
<td>MMP18</td>
<td>Collagenase 4</td>
<td>Helical collagen</td>
</tr>
<tr>
<td><strong>Gelatinases</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP2</td>
<td>Gelatinase A</td>
<td>Pro-MMP9, gelatin, elastin</td>
</tr>
<tr>
<td>MMP9</td>
<td>Gelatinase B</td>
<td>Gelatin, elastin</td>
</tr>
<tr>
<td><strong>Stromelysins</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP3</td>
<td>Stromelysin 1</td>
<td>Pro-MMP1, Pro-MMP7, Pro-MMP8, Pro-MMP9, Pro-MMP13, aggrecan, matrix components (incl fibrin)</td>
</tr>
<tr>
<td>MMP10</td>
<td>Stromelysin 2</td>
<td>Aggrecan, fibronectin</td>
</tr>
<tr>
<td><strong>Membrane-type MMPs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>MMP14</td>
<td>Pro-MMP2, Pro-MMP13, helical collagen, matrix components (incl fibrin)</td>
</tr>
<tr>
<td>MT2-MMP</td>
<td>MMP15</td>
<td>Fibronectin, aggrecan</td>
</tr>
<tr>
<td>MT3-MMP</td>
<td>MMP16</td>
<td>Collagen III, gelatin, fibronectin</td>
</tr>
<tr>
<td>MT4-MMP</td>
<td>MMP17</td>
<td>Unknown</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP7</td>
<td>Matrilysin</td>
<td>Pro-MMP9, aggrecan, matrix components (incl fibrin)</td>
</tr>
<tr>
<td>MMP11</td>
<td>Stromelysin 3</td>
<td>Serpin, weak activity for matrix components</td>
</tr>
<tr>
<td>MMP12</td>
<td>Metalloelastase</td>
<td>Elastin</td>
</tr>
<tr>
<td>MMP19</td>
<td></td>
<td>Gelatin</td>
</tr>
<tr>
<td>MMP20</td>
<td>Enamelysin</td>
<td>Amelogenin</td>
</tr>
</tbody>
</table>

Table 3. Detailing members of the MMP family. Fibrin is degraded by MMP3, MMP7, MMP9, MMP12 and MT1-MMP.

MMPs share significant sequence homology, and common multi-domain structure including a pro-peptide, a catalytic domain, a hinge and a haemopexin-like (PEX) domain (Johnson 1998; Seiki 2002). Some MMPs vary from this structure with additional insertions that presumably contribute to the functional difference between MMP types. As detailed in the table above (Table 3), MMPs are divided into four main classes, depending on their preferred...
substrate: collagenases, gelatinases, stromelysins, and membrane-type (Johnson 1998), however all MMPs are known to display overlapping but distinct substrate specificities and cell-specific expression (Shapiro 1998). Indeed, specific MMP knockout in transgenic animals has not been observed to affect the viability, development, fertility or life-span of the homozygous animals - indicating enough overlap of function within the MMP family to compensate for the loss of activity of a single member (Johnson 1998).

As a family of enzymes, the MMPs have been implicated in the remodeling of the ECM and the penetration of both normal and tumour cells through tissue barriers. Due to the high level of redundancy, however, it has been noted that no definitive single mechanism can be ascribed to all situations (Murphy 1999).

1.2.1.2.1 MT1-MMP and Other Fibrinolytic MMPs

A fine-tuned spatio-temporal regulation of the proteolytic activity is required for focused degradation of the ECM during extravasation, and membrane-anchored MMPs especially suited for pericellular proteolysis (Matias-Roman 2005). Following intracellular activation by furin-like enzymes, MT1-MMP is presented as an active enzyme anchored on the cell surface (Collen 2003). This apparatus restricts the function of these MMPs to the cells that produce them and causes them to regulate cellular functions in the tissue directly (Seiki 2002) - unlike most other MMPs, which, as secreted enzymes (Collen 2003), diffuse into the tissue cavity away from the producer cells.

MT1-MMP displays a wide range of proteolytic activities, including degrading cross-linked fibrin, fibrillar collagen, laminin-1, laminin-5, aggrecan, fibronectin and vitronectin. It has been seen to be important in tumour angiogenesis, development, bone formation and matrix degradation (Murphy 1995; Hiraoka 1998; Collen 2003; van Hinsbergh 2006). It is also known as the activating mechanism for pro-MM:P2 (in the presence of TIMP-2) and pro-MM:P13 (Cowell 1998; Lafleur 2001; Shankavaram 2001; Worley 2003). Furthermore, MT1-MMP processes cell adhesion molecules CD44 and pro-α5 integrin and tissue trans-glutaminase (van Hinsbergh 2006).

The membrane-bound proteolytic mechanisms of MT1-MMP and uPA-uPAR have much in common, and the membrane-anchored MMPs, specifically MT1-MMP, have been shown to be required during endothelial tubulogenesis within a fibrin matrix (Lafleur 2002). Like uPA-uPAR, MT1-MMP is directed toward the lamellipodia at the front of migrating cells,
suggesting an interaction between MT1-MMP and the actin cytoskeleton (van Hinsbergh 2006). Furthermore, VEGF-dependent activation of the uPAR-bound uPA activation was shown to be dependent on a change in integrin affinity as well as MT1-MMP-bound MMP2 activity on endothelial cells (Prager 2004).

The recent generation of MT1-MMP-deleted mice has provided much insight into its functions (Homlbeck 1999; Zhou 2000). It is interesting to note from experiments done in these mice that an additional subset of membrane-anchored MMPs, including MT2-MMP and MT3-MMP but not MT4-MMP allow cells to transverse fibrin matrices independently of MT1-MMP (Hotary 2002). These findings suggest a triad of MT-MMPs whose expression, alone or in combination, is sufficient to confer fibrin-invasive activity.

Other MMPs known to degrade fibrin include MMP3 and MMP7. MMP3 is also known as stromelysin-1, and its other substrates include fibronectin, laminin, collagens III, IV, IX, and X, and cartilage proteoglycans (Lijnen 2002). MMP7, also known as matrilysin, in addition to fibrin also degrades aggrecan, fibronectin, laminin, collagens III, IV, IX, and X, and cartilage proteoglycans. (Wilson 1997). Additionally, it has been shown that intestinal tumorigenesis is suppressed in mice lacking the metalloprotease matrilysin (Wilson 1997).

Both MMP3 and MMP7 are thought to be involved in wound repair, progression of atherosclerosis, and tumor initiation. Interestingly, both MMPs are known to activate pro-MMP9 (Lijnen 2002).

1.2.1.2.2 Gelatinases

A number of studies including gene deletions in mice have pointed to the essential role of MMP2 (gelatinase A) and MMP9 (gelatinase B) during the onset of angiogenesis in tumours and in development, bone formation and matrix degradation (Brooks 1998; Itoh 1998; Homlbeck 1999; Bergers 2000; van Hinsbergh 2006). They degrade a number of other proteins in the ECM, including gelatin and elastin. Although MMP2 and MMP9 were originally thought not to have direct fibrinolytic potential (Bini 1999), recent investigations have contradicted this observation (Hiraoka 1998; Lelonght 2001). One study, for example (Lelonght 2001), showed that in MMP9-null mice anti-glomerular basement membrane nephritis was accelerated, as attested by the extent of the fibrin deposits. These data strongly suggests that MMP9 is involved as a protective mechanism against the development
of these fibrin-induced glomerular lesions, through clearance or degradation of fibrin (Lelonght 2001).

Gelatinases also affect the release of some non-matrix substrates, including cell-surface and matrix-bound growth regulators which they release from ECM-sequestered stores (Chang 2001; van Hinsbergh 2006). Thus, the coordinated regulation of these MMPs and TIMPs has been seen to govern the cleavage and release of many important growth factors and cell surface receptors, such as TGF-β activation by MMP2 and 9, as well as FGFR-1 by MMP2 (Chang 2001). These findings further emphasise that during angiogenesis, MMPs can have both a pro-angiogenic role, by releasing matrix-bound pro-angiogenic factors, and also an anti-angiogenic role, by cleaving the ECM components into anti-angiogenic factors (Chang 2001; Mott 2004).

The switch from vascular quiescence to angiogenesis during carcinogenesis has recently been shown to involve MMP9-dependent release of VEGF-A from an extracellular reservoir (Bergers 2000; Mott 2004), suggesting that MMP9 is a component of the angiogenic switch (Bergers 2000). Notably, however, angiogenesis was observed to still occur when MMP9 was absent or functionally interfered with, which indicates that alternative regulatory mechanisms exist. Immunohistochemistry revealed that the MMP9 was expressed by a small number of cells in close apposition to the vasculature. These cells displayed features characteristic of infiltrating, inflammatory cells (Bergers 2000).

Immune cells, specifically macrophages, recruited to sites of allergic inflammation were seen to express MMP9 (Corry 2004) in mice. In studies done with MMP2 and MMP9 knockout mice, the researchers showed that despite similar levels of characteristic allergic and obstructive features, knockout and double knockout mice showed significantly reduced cells in total bronchoalveolar lavage, specifically eosinophils and neutrophils (Corry 2004). Lack of either MMP2 or MMP9 therefore did affect inflammatory cell trafficking and egression into the airway (Corry 2004; Greenlee 2006). It is proposed that both MMP2 and MMP9 are broadly implicated in inflammation by the establishment of multiple transepithelial chemokine gradients (McQuibban 2000; Greenlee 2006). These observations correlated with results obtained in a study of lavage fluid and sputum from patients with asthma where MMP9 expression was increased in patients with severe asthma, and the expression correlated with increased neutrophils and macrophages (but not eosinophils) (Wenzel 2003).
The release of pro-MMP9 by monocyte cell lines (THP-1 and U937 cells) into conditioned media increased under the action of recombinant single-chain urokinase. This effect occurred on both transcription and translational levels, yet was not accompanied by proteolytic activation of MMP9 (Menshikov 2004). This coupling of MMP9 expression to an element of the plasminogen/plasmin pathway further suggests that MMP9 expression is linked to fibrinolytic cascade.

The recent observations involving MMP9 have suggested that its function may be of significant importance in angiogenesis as well as aspects of inflammation, and may have effects over and above the degradation of specific substrates.

1.2.1.3 MMPs and Plasminogen/Plasmin

A complex interrelationship between the uPA/plasmin and the MMP systems is not only suggested by the fact that a number of MMPs (MMP1, 2, 3, and 9) depend on plasmin for their activation (van Hinsbergh 2006), but also by the fact that MMPs can control plasminogen activation by uPA-uPAR at the cell surface (Ugwu 1998; Collen 2003). This suggests that impairment of one protease system influences the activity of the other system. Indeed, it is thought that MMP versus plasmin-dependent fibrinolytic processes may be dictated by the repertoire of proteases available to the invading cell population (Hiraoka 1998). Increasingly, MMP activities are being shown to play crucial roles in proteolysis and cell migration. Endothelial cells at the leading edge of a new blood vessel concomitantly express components of both protease systems, and their expression is regulated by the same growth factors and cytokines (Collen 2003).

Extracellular proteolysis by the plasminogen/plasmin system and MMPs are required for tissue injury in autoimmune and inflammatory diseases (Liu 2005). In a study of bullous pemphigoid (BP), for example, the autoimmune disease which is characterized by dermal-epidermal separation, Liu et al (Liu 2005) showed that the plasminogen cascade is synergized with MMP9. Interestingly, mice deficient in MMP9 were resistant to experimental BP, while plasminogen-deficient, uPA-deficient, and tPA-deficient mice showed delayed onset BP. This suggests that, in this situation, the plasminogen/plasmin system is epistatic to MMP9 activation and BP onset.

Using plasminogen-deficient mice, Hiraoka et al demonstrated that the plasminogen/plasmin system is not necessary for neovascularization of fibrin by endothelial cells, but that MT1-
MMP was essential (Hiraoka 1998). This, however, is not consistent in other cell types, as keratinocytes, smooth muscle cells, and inflammatory cells all display major defects in their ability to invade or degrade fibrin deposits in plasminogen-deficient states in vivo (Hiraoka 1998). It would appear, thus, that different cell types rely variably on fibrinolytic mechanisms available.

These, and many other excellent studies suggest that the plasminogen/plasmin cascade and the MMP family of proteases cannot be considered as entirely separate systems, and that a hitherto unrealized amount of interaction exists between the two.

1.2.2 Integrins and Cell Adhesion

In addition to the vital role it plays in wound healing, cell migration also plays pivotal parts in a number of other biological events, including morphogenesis and tumour metastasis (Murphy 1999). The molecular mechanisms involved in effective cell migration include interactions between the cell and the ECM, cytoskeletal changes, cell signaling and gene regulation. Because of the wide range of processes involved, cell migration is therefore influenced not only by the cell type, but also by variables such as their origin, adhesion receptor function and environment, including the nature of the ECM (Murphy 1999). The complex response of cell migration requires the coordination and cooperation of multiple cell surface receptors, including sensory receptors that detect migratory stimuli, adhesion receptors that mediate interactions of migrating cells with the ECM, and protease receptors that facilitate movement of cells through their extracellular environment (Pluskota 2003).

As transmembrane glycoproteins, integrins can bind ECM components and trigger signal transduction cascades (Clark 2003). On the cell surface integrins ligands include growth factors and MMPs, while inside the cell integrin-triggered signal pathways integrate with those emanating from growth factor ligation. Integrins are heterodimeric cell surface adhesion receptors formed by two non-covalently associated subunits, α and β (Hynes 1992; Hynes 1999; Stupack 2002; Ruegg 2004), which mediate cell-cell and cell-matrix adhesion (Ahlen 2004). These subunits form αβ combinations with unique binding specificity and signaling properties (Demetriou 2004). Integrins connect extracellular matrix fibers with the actin cytoskeleton at focal adhesion sites, and integrate and govern interacellular signaling events (Ahlen 2004). They have been shown to recognize ECM proteins, such as fibronectin, laminins, collagens or vitronectin, and allow the cell to sense the ECM environment. Integrins are known to be involved in many different processes, including cell survival,
migration and differentiation, as well as blood clotting and tissue organization (Ruegg 2003; Demetriou 2004; Ruegg 2004).

Importantly, integrins are known to be promiscuous, and one integrin can bind to several different ligands, while, conversely, a number of different integrins can bind the same ligand (Ruegg 2004). Redundancy may be an advantage when the cellular response (such as survival or migration) in a particular context is more important than the identity of the particular ECM protein eliciting the response (Hynes 1992; Ruegg 2004). This is important in situations such as wound healing or tissue remodeling, when resident and infiltrating cells have to rapidly react and adapt to changes in the composition of the ECM. As integrins have no intrinsic enzymatic activity, signal transduction depends on the recruitment of cytoplasmic structural and signaling proteins and the assembly of characteristic structures named focal contacts and focal adhesions (Ruegg 2004). Importantly, many of these signaling pathways that are activated by integrins are also known to be activated by growth factor receptors, and cross-talk between these two elements is believed to provide enhanced specificity and control over many different cellular events (Ruegg 2004). Integrins stimulate 4 major pathways that are involved in angiogenesis: the mitogen activated protein kinase (MAPK) pathway, the phosphoinositide 3-kinase (PI-3-K)-PKB/Akt pathway, Rho family GTPases and NF-κB (Ruegg 2004).

Both serine and metalloproteinases have been implicated in the complex integrated events underlying cell migration but no definitive single mechanism has emerged (Murphy 1999). The molecular mechanisms behind the various cellular strategies employed are being analysed at all levels, from interactions between the cell and its extracellular matrix, cytoskeletal changes, cell signaling, and gene regulation. The efficient integration of these processes is a key determinant of different cell migration patterns and a major challenge to a rational understanding of events.

1.3 Monocytes and Macrophages

1.3.1 Monocyte Morphology and General Characteristics

Circulating monocytes are mononuclear phagocyte precursors to tissue-resident macrophages, as well as to other specialized cells, such as dendritic cells and osteoclasts (Satchura 1989; Shannon 2003; Gordon 2005). They are derived from multipotent stem cells in the bone marrow, and form 5% of the total leukocyte population - in keeping with their
role in chronic rather than acute inflammatory responses (Duffield 2003). Once formed, they are released into the blood and circulate in the bloodstream for an average of 1–2 days, before they enter the tissues and mature into macrophages (Satohura 1989; Duffield 2003; Gordon 2005).

Macrophages are specialized phagocytic cells able to adapt to a great variety of cellular and microbial stimuli, and are known to secrete a wide range of molecules, including various cytokines. They are distributed throughout the tissues of the body, and play crucial roles in both the innate and acquired immune systems (Gordon). During inflammation and repair macrophages demonstrate activities that include regulating inflammatory cells, tissue debridement, cell killing, recruiting and activating myofibroblasts and regulating spontaneous recovery of fibrosis in physiological or aberrant wound healing (Friedberg 1974; Kodelja 1997; Duffield 2005).

As circulating macrophage-precursors, however, monocytes lack the phagocytic capacity of differentiated macrophages, and their cell surface receptor/ligand complement renders them relatively inert whilst in the blood compartment (Duffield 2003). Upon injury in vivo, monocytes, as other leukocytes, are recruited to the cytokine-activated endothelium in a multistep process. The monocytes in the bloodstream are slowed down by a capturing mechanism and roll over the endothelial layer. Activation of the monocytes during this rolling phase results in firm adhesion and transmigration through the endothelial cell layer (Loschinsky 1994; da Costa Martins 2006). They rapidly cross the endothelial barrier using selectins, integrins and other receptors of the immunoglobulin superfamily (Albeda 1994; Duffield 2003). Once inside the inflamed site, chemokines and cytokines released from activated endothelial, epithelial and mesenchymal cells signal to the naïve monocyte and starts its differentiation into the macrophage (Duffield 2003). This activation involves a number of changes, as detailed in the table below (table 4).
During activation, monocytes experience enhanced cellular metabolism, mobility, lysosomal enzyme activity and cytocidal capacity (Ross 2002). This is usually accompanied by increased elaboration of important products of mononuclear phagocytes, including lysosomal neutral proteases, acid hydrolases, complement components, enzyme inhibitors, binding proteins, interleukin (IL)-1, and factors promoting haematopoiesis.

Recent cell surface phenotyping studies have suggested that distinct sub-populations of macrophages exist (Duffield 2005). Depending on expression of receptors, such as CD163, two forms of inflammatory macrophages have been proposed: the classically and the alternatively activated macrophage (Gordon 1999; Duffield 2005), although these classifications may still be a simplification of the in vivo functions of macrophages. Alternatively activated macrophages produce anti-inflammatory cytokines, including IL-10 and TGF-β. When cultured with myofibroblasts, alternatively activated macrophages promote complex matrix deposition. Classically activated inflammatory macrophages, on the other hand, play crucial roles in bringing about matrix destruction through the production of MMPs (Duffield 2003). This matrix degrading proteolytic activity is thought to allow cell-killing monocyte/macrophages to migrate easily through matrix structures, where they are essential for the first phase of wound healing through the containment and clearance of debris.

Monocytes are recruited to non-immune sites of inflammation following metabolic and other forms of persistent injury by a variety of different signals (Gordon), and are one of the first
cell types to migrate into the fibrin clot (Henderson 2003; Moldovan 2005) - within the first two hours of inflammation - responding rapidly to signals produced in situ at the site of inflammation (Henderson 2003), such as thrombin (Bar-Shavit 1983). This recruitment in response to activating and chemotactic factors is thought to be a key event during the inflammatory response (Henderson 2003; Matias-Roman 2005). Interestingly, while the rapid recruitment of inflammatory monocyte migration is accompanied by neutrophil migration, these processes have been shown to be independent or each other (Henderson 2003).

Despite being known to release a number of agents that assist in the degradation of the matrix, and although in almost all instances of adult angiogenesis the growth of new vessels is associated with inflammation (Dvorak 1995), the precise role of monocyte/macrophages at the site of injury remains unclear due to conflicting experimental results (Duffield 2005). Some studies have shown monocyte depletion from the angiogenic environment resulting in impaired angiogenesis, suggesting interdependence between monocyte inflammation and angiogenesis (Leibovich 1975; Jackson 1997; Croll 2004; Moldovan 2005). Indeed, activated monocytes and/or macrophages alone were sufficient to induce angiogenesis in the avascular cornea, probably through the elaboration of growth factors and cytokines known to regulate angiogenesis (Jackson 1997).

A study done with L-selectin and intercellular adhesion molecule-1 (ICAM-1) knockout mice showed delayed wound healing manifested by decreased wound leukocyte (monocyte/macrophages and neutrophils) accumulation (Nagaoka 2000). L-selectin is constitutively expressed by most leukocytes and mediate leukocyte capture and rolling along the endothelium (Tedder 1995), while ICAM-1 is expressed by ECs and is involved in leukocyte-endothelial cell adhesion (Dustin 1986). These results suggest that leukocyte infiltration into the wound is necessary for healing. Treatment of the wounds with PDGF normalized delayed wound healing in ICAM-1−/− mice, but not in L-selectin/ICAM-1−/− mice.

Other investigations, however, have recently raised the suggestion that the steroid treatment in some of the depletion studies may have knocked down more than just leukocytes at the wound site. Wound healing studies in the PU.1 null mouse (Martin 2003), which is genetically incapable of raising the standard inflammatory response because it lacks macrophages and functioning neutrophils, have shown that these mice are capable of scar-free wound repair in similar time to wild-type mice. These results suggest that the inflammatory response is not absolutely essential for tissue repair. Another study using
CD11b-DTR transgenic mice, in which macrophages could be selectively depleted, suggested that macrophage depletion during advanced liver fibrosis resulted in reduced scarring and fewer myofibroblasts. In contrast, monocyte depletion during recovery led to a failure of matrix degradation. This study suggested that distinct sub-populations of macrophages exist in the same tissue and play different roles in both injury and repair (Duffield 2005). It is of importance however, that in these studies, despite the convincing wound healing data, it must be noted that the level of angiogenesis was not investigated under the experimental conditions.

That the inflammatory macrophage is able to mediate the destruction of the matrix and mediate apoptosis, yet also possesses the ability to aid cell proliferation and secrete new matrix components, suggests that the role of monocyte/macrophages in wound healing is complex (Duffield 2003). The recent discovery of different sub-populations of inflammatory macrophages also suggests more diverse roles than previously suspected (Duffield 2003; Duffield 2005).

1.3.2 Monocyte Fibrinolysis and Invasive Behaviour

Despite the ambiguous role of monocytes in wound healing, during tissue repair, pathological remodeling or tumoural growth, an inflammatory infiltrate, composed mainly of monocytes/macrophages, precedes or accompanies angiogenesis (Moldovan 2005). Monocytes are recruited in response to many different stimuli. One of the major cytokines known to influence monocyte chemotaxis is VEGF (Croll 2004; Murdoch 2004). Monocyte chemotactic protein-1 (MCP-1), TGF-α and TGF-β are also known to modulate monocyte infiltration (Moldovan 2005).

Upon extravasation from the vasculature, monocytes encounter the extracellular matrix, which leads them to adopt an angiogenic phenotype (White 2001; Levi 2004). In particular, fibronectin has been shown to be a potent inducer of angiogenic activity in monocyte (signaling via the integrin αβ1). Accompanying endothelial transmigration, monocytes must traverse tissue barriers composed of distinct structural proteins (Moldovan 2003; Matias-Roman 2005), and a provisional matrix of fibrin deposited during vascular injury. Therefore, invasion into the wound area necessitates that the ability to degrade the ECM is an essential requirement for monocyte transmigration, and requires the proteolytic action of specific enzymes (Matias-Roman 2005). The major fibrinolytic pathway involves the activation of plasminogen by the endogenous plasminogen activators uPA and tPA (Loscalzo 1996).
Monocytes possess fibrin(ogen) receptors and migrate intravascularly to sites of fibrin deposition during the inflammatory response (Simon 1996).

In plasminogen-deficient mice, however, plasmin-independent mechanisms may account for up to 33% of clot lysis (Ploplis 1995; Simon 1996). These plasmin-independent fibrinolytic pathways in leukocytes also include both the extracellular release of fibrinolytic proteases (Simon 1996; Moldovan 2000) and the phagocytosis of fibrin (Loscalzo 1996; Simon 1996). MMP studies conducted by Moldovan et al (Moldovan 2000) have described monocytes/macrophages traveling through fibrin in a manner which they describe as “MMP12-dependent-tunnelling”. It has been suggested that these “tunnels” form pathways for endothelialization by later migrating endothelial cells (Moldovan 2000). Other MMP studies have shown monocytes to produce MMP1, MMP3, MMP9, MMP10, MT1-MMP, MMP19 and MMP25 (Bar-Or 2003), of which MMP3 and MT1-MMP are important fibrinolytic MMPs. Fibrin degradation has also been seen to occur by way of a proteolytic system within the macrophage lysosome that does not involve plasmin. This alternate pathway involves first the binding of fibrin(ogen) to the surface integrin αβ₂ (CD11b/CD18) followed by internalization of the complex into the lysosome where the protease cathepsin D degrades the protein (Loscalzo 1996). Furthermore, in an interesting study, Ciano et al demonstrated that the occurrence of tunnels in fibrin gels was dependent on the density of the fibrin matrix, and monocytes/macrophages were able to push apart the fibrin strands in lax gels, thereby moving through the fibrin gel without fibrinolysis (Ciano 1986; Lanir 1988).

1.3.2.1 Monocytes and the Plasminogen/Plasmin System

Fibrinolytic factors, in particular uPA and uPAR, may modulate the inflammatory response by their effect on inflammatory cell recruitment and migration (Levi 2004). uPAR is known to mediate leukocyte adhesion to the vascular wall or ECM components, and the expression of uPAR on leukocytes is strongly associated with their migratory and tissue-invasive potential (Blasi 1997). In migrating monocytes uPAR is expressed in the microenvironment of the leading edge, producing a “front” of proteolysis that facilitates degradation of fibrin thrombus or ECM, and the expression of uPAR on leukocytes is strongly associated with their migratory and tissue-invasive potential (Blasi 1997; Levi 2004; Murdoch 2004). Indeed, the recruitment of mononuclear cells to infarcted areas in patients with myocardial infarctions is connected with enhanced uPAR expression on the surface of these cells. The underlying mechanism by which uPAR and uPA affect cell migration may be related to ECM degradation by proteases that are activated by uPAR-associated uPA (such as MMPs).
Human monocytes are also known to produce tPA, PAI-1, PAI-2 in addition to uPA and uPAR (Loscalzo 1996; Pierleoni 2003).

Studies in knockout mice have also shown monocyte-expressed uPAR to exert protease-independent properties, involving transmembrane signal transduction after interaction with proteins or receptors, which leads to cytokine and growth factor production (Blasi 2002; Levi 2004). In those models, observations suggest that the function of uPAR in chemotaxis was independent from its interaction with uPA, and interference of uPAR binding to ligands or modulation of uPAR-dependent cell signaling were seen to affect leukocyte recruitment and invasion in inflamed tissue areas as a result of infarction (Levi 2004). The extensive involvement of uPAR in signaling has lead to the suggestion that the type of ligand for uPAR determines gene expression by this receptor in monocytes (Rao 1995).

uPAR is known to be a multifunctional protein capable of not only promoting peri-cellular proteolysis and matrix attachment, but also effecting cysteine- and MMP expression during macrophage differentiation (Rao 1995). Indeed, uPAR is thought to be one of the markers of monocyte activation (Pierleoni 2003). In addition, uPA not only promotes pericellular proteolysis, but can also directly influence the expression of cathepsin-B and MMP9 mRNA and protein during macrophage differentiation (Menshikov 2004). These results extend the cooperation seen among distinct classes of proteases at the macrophage cell surface to regulation of their gene expression (Rao 1995).

1.3.2.2 Monocytes and MMPs

In a 2003 study, Bar-Or et al utilized quantitative real time PCR to systematically analyse the expression of all 23 MMP members in subsets of leukocytes isolated from the blood of normal individuals. In monocytes MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-10, MT1-MMP, MMP-19 and MMP-25 were all significantly represented. Interestingly, MMP-2 and MT1-MMP were both upregulated in patients with multiple sclerosis, suggesting their involvement in monocyte transmigration (Bar-Or 2003).

MT1-MMP is known to be involved in monocyte migration (Matias-Roman 2005) and has been observed to be regulated through the cell's interaction with fibronectin or the endothelium. MT1-MMP clustering was observed at motility-associated membrane protrusions of MCP-1-stimulated monocytes migrating. In addition, upregulation of MT1-MMP expression was induced in monocytes upon attachment to fibronectin in a manner
dependent on integrins α4β1 and α4β1. These observations suggest that MT1-MMP has a key role in monocyte recruitment during inflammation.

It has been suggested that freshly isolated monocytes produce primarily serine proteinases, but become predominantly MMP-producing cells when cultured on plastic in the presence of serum in vitro (Lepidi 2001). Indeed, the upregulation of MMP9 expression is commonly taken as a marker associated with monocyte differentiation (Menshikov 2004). Furthermore, the analysis of the temporal pattern of MMP expression during the healing of skin wounds in the mouse showed that after an initial outburst of more common collagenases and of their inhibitors, the later stages of healing were characterized by macrophage-specific MMP (MMP-12) expression. It was observed that this expression was dominant when the cells were clustered around vascular structures (Anghelina 2002; Moldovan 2005).

1.3.2.3 Monocytes and Integrins

Integrins are surface receptors which interact with the cytoskeleton, and integrate the extracellular environment with the cell interior, while also able to induce functional changes in the cell by activating intracellular biochemical signaling cascades (Reyes-Reyes 2002). They have been found to participate in many aspects of the inflammatory response, especially in cell migration, adherence and activation (Petty 2002).

Members of the β2 integrin family are the dominating integrins expressed on leukocytes, known to play major roles in leukocyte cell-cell and cell-matrix adhesions during inflammation and other immune responses (Dib 2000). One of the earliest events in β2 signaling is the activation of non-receptor tyrosine kinases, which in turn trigger downstream activation of various signaling pathways that affect different functional responses of the cell. These non-receptor tyrosine kinases include the src family, Syk, focal adhesion kinase (FAK), and fakB, Rac and Rho (Eliceiri 2001).

In addition to the β2 integrin family, monocytes also express a number of other integrins, including the β1 integrins (Arnaout 1990; Reyes-Reyes 2002). While β2 integrins mediate cell-cell interactions that are important for migration through the endothelium, and also for phagocytosis, the β1 family mediates adhesion to extracellular matrix proteins, which results in a strong induction of immediate early genes that are important in inflammation (Reyes-Reyes 2002). Therefore it is evident that the β1 and β2 families of integrins regulate different leukocyte cell processes during inflammation. Interestingly, Reyes-Reyes et al showed that
the β1 and β2 integrin families, while sometimes resulting in the same outcome, activate different signaling pathways (Reyes-Reyes 2002). For example, adhesion of human blood monocytes to ECM proteins as well as ligation of the β1 integrins with antibodies results in activation of the nuclear transcription factor NF-κB through PI-3K (Reyes-Reyes 2002), which is required for the transcription of inflammatory genes. β2 integrins, on the other hand, while also able to activate NF-κB, did not use the PI-3K pathway to do so (Reyes-Reyes 2002).

Interestingly, many of the signaling pathways and effectors which are activated by integrin ligation are also activated after growth factor stimulation (Eliceiri 2001). Growth factor-induced cell proliferation, adhesion and migration in cultured cell models involving factors such as PDGF, bFGF, VEGF, and endothelial growth factor (EGF) often depends on specific integrins (Miyamoto 1996; Eliceiri 2001; Grundstrom 2003). In cooperation with aggregated, ligand-bound integrins, these growth factors produced a marked, transient activation of the extracellular signal-regulated kinase (ERK) class of MAP kinase (Miyamoto 1996). Interestingly, the growth factor receptors were induced to aggregate transiently by integrin ligand-coated beads in a process requiring both aggregation and occupancy of integrin receptors, but not necessarily the presence of growth factor ligands (Miyamoto 1996). Thus, the assembly of integrins and growth factor receptors into large signaling and cytoskeletal complexes, after matrix contact involving both integrin aggregation and occupancy, could explain the synergy observed when effectors and substrates are transiently concentrated together at a local site for greater mutual interaction (Miyamoto 1996).

The plasma membrane receptors known to physically interact and functionally cooperate with integrins includes uPAR and PDGF receptor-β (PDGFR-β) (Plopper; Sundberg 1996; Petty 2002), resulting in signaling pathways converging within focal adhesion complexes (Plopper 1995). Multiple signaling molecules, including PI-3K, involved in both integrin and growth factor receptor signaling pathways became associated with the cytoskeletal framework of the focal adhesion complex after integrin ligand adhesion (Plopper 1995). Tyrosine kinase receptors were also recruited to the focal adhesion complex, and tyrosine phosphorylation was enhanced (Plopper 1995; Reyes-Reyes 2002). Interestingly, recent investigations have also shown cell-surface association between MMPs and β2 integrins was seen to influence neutrophil migration and cancer progression (Stefanidakis 2006), suggesting that leukocyte motility is dependent on both β2 integrins and the gelatinases MMP2 and MMP9.
The $\beta_2$ integrins (also known as the CD11/CD18 family) consists of three different receptors which all share a common $\beta_2$ unit, while having different $\alpha$ units (Arnaout 1990; Dib 2000). The family includes integrin $\alpha_L\beta_2$ (CD11a/CD18), which is present on all leukocytes, integrin $\alpha_M\beta_2$ (Mac-1, CD11b/CD18) and integrin $\alpha_P\beta_2$ (CD11c/CD18), which are restricted to monocytes, macrophages, neutrophils and natural killer cells (Arnaout 1990). The expression of these integrins is dependent on the cell type and the state of cell activation and differentiation (Arnaout 1990). In resting monocytes, for example, the expression of $\alpha_P\beta_2$ is roughly equivalent to that of $\alpha_M\beta_2$, and both are greater than $\alpha_L\beta_2$. Upon activation, the cell surface expression of these integrins increases from the translocation of intracellularly-stored pools.

The multivalent binding properties of the leukocyte integrin $\alpha_M\beta_2$ allows this receptor, amongst other things, to regulate alternative pathways of fibrin degradation (Simon 1996). Indeed, as mentioned above, after activation integrin $\alpha_M\beta_2$ is able to bind fibrin(ogen) (Altieri 1986), which facilitates the internalization and degradation of fibrin via cathepsin D (Simon 1993; Loscalzo 1996). Interestingly, the ligation of integrin $\alpha_M\beta_2$ to fibrin also results in the release of neutrophil elastase, which has fibrin degrading properties (Weitz 1987). Furthermore, integrin $\alpha_M\beta_2$ and uPAR have been shown to form a functional unit on monocyte cells (Simon 1996). Together they mediate complementary functions and promote the degradation of fibrin(ogen) and confer adhesive properties on the cells (Simon 1996; Sitrin 1996). In this situation, uPAR occupancy by uPA inhibits integrin $\alpha_M\beta_2$-mediated fibrin degradation by monocytes. Thus, it has been suggested that where relatively large amounts of fibrinolytic activity could be expected, $\alpha_M\beta_2$ function appears to be downregulated. Conversely, under conditions where little plasmin is being generated by uPAR, the receptor functions to promote $\alpha_M\beta_2$ activity (Simon 1996). These results imply a functional coupling of $\alpha_M\beta_2$ and uPAR, which operates to regulate the interaction between monocytic cells and the components of the fibrinolytic cascade. In addition, $\alpha_M\beta_2$-mediated adhesion to fibrinogen is dependent on uPAR (Sitrin 1996; Zhang 2003), and adhesion is increased by occupancy of uPAR with uPA. Phosphorylation of FAK and MAPK was increased by the interaction of uPAR with $\alpha_M\beta_2$.

Direct phosphorylation of integrin cytoplasmic stalks can mediate platelet aggregation (Law 1999). Human monocytes adhere to activated platelets, and form platelet-monocyte complexes (PMC) (da Costa Martins 2006), in an P-selectin- and P-selectin glycoprotein ligand-1 (PSGL-1)-dependent manner. Monocytes within these PMC have increased adhesive capacity to the activated endothelium. It was recently shown (da Costa Martins...
2006) that the P-selectin-PSGL-1 interaction induced increased expression and activity of integrins \( \alpha \beta_1 \) and \( \alpha \beta_2 \). The formation of these PMC also increased monocyte transendothelial migration. These data suggested that by binding to platelets, monocytes displayed increased adhesion to the activated endothelium, and were in a higher state of activation (da Costa Martins 2006).

1.4 Angiogenic Cytokines

Cytokines are soluble non-immunogloblin (glyco)proteins which are released by living cells, and act non-enzymatically in pico-molar to nano-molar concentrations to regulate host cell function. They make up the fourth major class of soluble intercellular signaling molecules, alongside neurotransmitters, endocrine hormones and autacoids (Nathan 1991). Cytokines play central roles in a number of different processes, including the (re)modeling of tissues, angiogenesis and inflammation. The two cytokines focused on in this study – Ang-2 and PDGF-BB are discussed in detail below. These two factors were chosen for their spatiotemporal co-expression with monocytes during early wound healing.

1.4.1 Platelet-Derived Growth Factor-BB (PDGF-BB)

1.4.1.1 Overview of Functions

Members of the PDGF family are a family of polypeptide growth factors that affect a number of cell types (Antoniades 1979; Hoyle 1999). They were originally characterized as the predominant mitogen in serum, (Antoniades 1979; Hoyle 1999; Hoch 2003), and have since been shown to drive cellular responses including proliferation, survival, migration and the deposition of ECM, and tissue remodeling factors (Schwartz 1990; Bornfeldt 1994). The severity of the various PDGF and PDGFR knockout mice phenotypes indicate pleiotrophic involvement in development, and it is only through the use of conditional gene ablation transgenics that some roles of PDGF and PDGFR could be elucidated (Hoch 2003).

The PDGF family is made up of four ligands: PDGF-A - D, and two tyrosine kinase receptors: PDGFR-\( \alpha \), and PDGFR-\( \beta \). All PDGFs can function as secreted, disulfide-linked homodimers, while PDGFA and B can also form functional heterodimers (Siegbahn 1990; Hoch 2003). Upon ligand dimer binding, the receptors dimerize, activate, and initiate signal transduction in, among others, the Ras-MAPK, PI3K, and phospholipase C\( \gamma \) pathways (Hoch 2003).
One of the sources of serum-derived PDGF-BB are megakaryocytes, and PDGF-BB is stored (along with other PDGFs and other substances) in the α-granules of human platelets (Hoyle 1999), and released upon degranulation (Hosgood 1998; Beer 2000). It is also produced by a number of cell types, including macrophages, epithelial, endothelial, mesenchymal, and neuronal cells (Hosgood 1993; Hoyle 1999).

In order to maintain the ability to elicit pleiotrophic effects, expression of the PDGF family is tightly controlled. Although there are slight differences between the signaling molecules activated by the α and β receptors, major differences between the functions of the receptors derive from their expression patterns (Funa 2003). Cellular responses to PDGFs have also been seen to differ at distinct times or locations during development, and it is possible that other signals influence PDGF-mediated responses (Hoch 2003). This suggests that not only the expression of PDGF receptors and ligands, but also the cell differentiation state of the cell and local signaling environment play factors in determining PDGF-mediated responses. As the PDGF family has also been seen to interact with other cytokines (Bornfeldt 1994), such as bFGF (Russo 2002), the growth factors present in the extracellular milieu contribute to the effect of PDGF on cells.

1.4.1.2 PDGF-BB/PDGFR-β and Accessory Factors

Following receptor ligation by PDGF-BB, the PDGFR-β dimerizes, undergoes autophosphorylation, and recruits a diverse set of at least 10 SH2 domain-containing proteins that then activate multiple downstream cascades which serve to control a number of different cellular functions (DeMali 1999; Lehti 2005). Independent of this action, PDGF-B/PDGFR-β effector function can be further modulated by interactions with accessory factors including growth factors and cell adhesion molecules (Schneller 1997; DeMali 1999; Lehti 2005).

The PDGF family is known to associate with a number of different integrins, which has lead to the suggestion that integrin-mediated signaling processes synergize with growth factor responses. Integrin αβ3, for example, associates with activated insulin and PDGFR-β and potentiates the biological activity of PDGF in fibroblasts (Schneller 1997). Also in fibroblasts, PDGF-BB has been shown to modulate membrane motility of β1 integrins (Ahlen 2004).

In monocyte cell lines RhoA and integrin clustering were activated by GM-CSF, MCP-1 and PDGF-BB in human monocytic cell lines (Kohno 2004), suggesting that cell migration was...
mediated by the association. Treatment of VSMC with PDGF-BB significantly increased binding to human monocytic THP-1 cells and to peripheral blood monocytes, and was inhibited by antibodies to monocyte β1 and β2 integrins (Cai 2004). This effect was shown to involve Src, PI-3-K, and MAPK.

A number of studies have been conducted on PDGF-BB interaction with elements of the fibrinolytic cascades. For example, a study by Lehti et al using MT1-MMP-deficient mice determined that MT1-MMP is a PDGF-BB-selective regulator of PDGFRβ dependent signal transduction and mural cell function. Catalytically active MT1-MMP associates with PDGFR-β in membrane complexes that support the efficient induction of mitogenic signaling by PDGF-BB in an MMP inhibitor-sensitive fashion, facilitating chemotaxis and proliferation as well as ERK1/2 and Akt activation (Lehti 2005). Furthermore, it was observed that PDGF-B/PDGFR-β function can be further modulated by interactions with accessory factors ranging from non-PDGF peptide growth factors and sphingolipids in addition to the cell adhesion molecules (DeMali 1999; Lehti 2005). While fibrinolysis was not investigated in these studies, it is highly possible that PDGFR-β, associated as it is with MT1-MMP, would influence mural cell fibrinolysis.

uPAR activation in VSMC by uPA induces its association with PDGFR-β. The interaction results in PDGF-independent PDGFR-β activation and dimerization, and the mediation of uPA-induced downstream signaling relating to migration and proliferation (Kiyan 2005). Furthermore, PDGF and bFGF have also been shown to increase uPA expression in VSMC (Padro 2002), and uPA, in turn, mediates the mitogenic activity of the growth factors in a catalytic-domain-dependent manner.

Interestingly, a correlation has been established between PDGF-BB expression and lung fibrosis in both human disease and in animal models (Hoyle, 1999). Pdgfb transgenic overexpression in the lung gave rise to emphysema in adult mice, and these animals display thickened septa, enlarged saccules, severe inflammation and fibrosis. Knockout and transgenic studies showed that overexpression of PDGF-BB resulted in localized areas of fibrosis that exhibited increased cellularity and ECM deposition. The focal nature of the fibrosis, however, suggested that PDGF-BB overexpression by itself could not produce fibrosis, but that additional events were required to trigger the fibrotic phenotype (Hoyle 1999).
The pleiotrophic effects of the PDGF family, and the observations that PDGF and PDGFR influenced, and influence, the activity of various elements of the fibrinolytic system, suggest that PDGF may be more closely involved in fibrinolysis than previously thought.

1.4.1.3 PDGF and Inflammation

PDGFR-β has previously been reported to be expressed on monocytes (Kumagai 2001), and chemotaxis studies in vitro have shown PDGF-BB to be chemotactic for monocytes (Siegbahn 1990; Kohno 2004). Indeed, in transgenic mice overexpressing PDGF under the lung-specific surfactant protein-C promoter, inflammation composed mainly of monocyte/macrophages and eosinophils was always observed within or adjacent to fibrotic regions in the lung - an observation consistent with the findings from human interstitial lung disease, and in other animal models (Hoyle 1999).

PDGF-BB delivery into chronic non-healing wounds has been seen to exaggerate the inflammatory and proliferative/matrix deposition phases of repair (Pierce 2001). Additionally, PDGF-BB has also been seen to interact with other elements of the wound healing process. For example, PDGF-BB and MCP-1 have been observed to induce TF expression in human peripheral blood monocytes (Ernofsson 1996), the initiator of the clotting cascade. This observation suggests a PDGF-BB-dependent link between haemostasis and inflammation.

1.4.2 Angiopoietin-2 (Ang-2)

1.4.2.1 Ang-2 and Angiogenesis

Angiopoietin-2 (Ang-2) was first identified by Maisonpierre et al in 1997 using homology screening (Maisonpierre 1997). It is a growth factor belonging to the angiopoietin family (composed of Ang-1 to Ang-4), and is structurally related to Ang-1, with which it shares the tyrosine kinase receptor Tie-2 (Lemieux 2005).

Tie-2 was initially thought to be an endothelial-specific receptor, however recent investigations have shown its presence on a variety of other cells (Koh 2002; Iurlaro 2003; Lemieux 2005). Tie-2 is unique among receptor tyrosine kinases in that its extracellular ligands, the angiopoietins, appear to have opposing actions on endothelial cells, despite sharing around 60% amino acid sequence identity (White 2003). Ang-1 acts as an activating
ligand that induces phosphorylation of Tie-2 and promotes endothelial cell survival, vascular impermeability (Fiedler 2006) and integrity (Jones 2003). Ang-1 is also known to play both anti-permeability and anti-inflammatory roles by decreasing both adhesion of leukocytes to the endothelium, as well as strengthening cell junctions through the localization of PECAM-1 (platelet-endothelial cell adhesion molecule-1) (Jones 2003). Ang-2, on the other hand, acts as a steric inhibitor of Ang-1’s action on Tie-2 (Maisonpierre 1997).

By binding to Tie-2, and blocking its Ang-1-mediated activation, Ang-2 causes the destabilization of the endothelium and vessel disruption (Kampfer 2001; Fiedler 2006). Through the mediation of the endothelium into a more plastic state, Ang-2 is believed to potentiate the actions of pro-angiogenic growth factors, such as VEGF and bFGF (Lobov 2002; Visconti 2002; White 2003). Indeed, exogenous Ang-2 has been shown to enhance VEGF-mediated corneal neo-vascularization (Lobov 2002; Asahara 2003), while ectopic over-expression of Ang-2 in cancer cell lines produced larger, more vascular tumours in mice.

Ang-1 is constitutively expressed at low levels in the quiescent adult vasculature (White 2003). In contrast, Ang-2 appears to be expressed only at sites of active angiogenesis, such as the ovary, vascularized tumours and sites of injury (Maisonpierre 1997; Kampfer 2001). While matrix-associated Ang-1 is thought to act in a paracrine agonistic manner, Ang-2 appears to primarily act in a diffuse, autocrine antagonist regulatory manner to control endothelial cell quiescence and responsiveness (Fiedler 2004; Dallabrida 2005). In keeping with the observation that Ang-2 is expressed only at sites of active angiogenesis (Maisonpierre 1997), a number of different angiogenic inducers are known to upregulate Ang-2 expression. These include hypoxia, cyclooxygenase-2 (COX-2), VEGF, bFGF, TNF-α, leptin, prostaglandins (PGEs and PGI₃) (Pichiule 2004), HIF-1α, the von Hippel-Lindau gene, angiotensin II, oestrogen, and HER2 (Koh 2002; Jones 2003). The diversity of these inducers seems to suggest that the upregulation of Ang-2 might be a common pathway by which different angiogenic inducers act (Koh 2002; Cai 2003).

Transgenic studies have shown the essential role of Ang-1 in vascular (re)modeling (Jones 2003), and Ang-1-null mice die at embryonic day 12.5 showing defects in their vasculature. Studies using Ang-2-knockout mice, however, show that loss of the gene and function is compatible with life (Gale 2002). Ang-2-deficient mice are born relatively normal with only minor abnormalities in the vascular system, which do not affect function. Interestingly, however, in some strains of Ang-2-deficient mice (129/J), newborn pups die at 14d from chylous ascites, and it has been noted there are defects in the hyaloid vessels, lymphatic
vessels and kidney cortical peritubular capillaries (Jones 2003; Fiedler 2006). In contrast, mice transgenically overexpressing Ang-2 have an embryonic lethal phenotype, which is similar to both Ang-1- and Tie-2-deficient phenotypes (Sato 1995; Suri 1996; Maisonpierre 1997).

Studies with transgenic mice do however suggest that Ang-2 functions cannot be limited to an Ang-1 antagonist, and Ang-2 has been observed to act as a Tie-2 agonist in postnatal remodeling events. In vivo studies using either the rabbit corneal pocket assay (Asahara 2003) or the murine papillary membrane (Lobov 2002) also show that Ang-2 can act as a pro-angiogenic factor. This suggests that the function of Ang-2 may be more dependent on the context in which it is found than has previously been understood. Furthermore, in vitro research has shown Ang-2 to be able to activate Tie-2, stimulate EC migration, and stimulate EC capillary-like tube formation in vitro (Mandriota 1998; Teichert-Kuliszewska 2001; Lemieux 2005). It has also been seen to stimulate migration and tube-like structure formation of murine brain capillary endothelial cells through c-Fes and c-Fyn (Mochizuki 2002).

When acting in the absence of VEGF, however, Ang-2 has been shown to stimulate apoptosis and vascular regression in capillary endothelial cells (Lobov 2002) - in contrast to its migratory and proliferatory roles in the presence of VEGF (Mandriota 1998). Furthermore, the inhibition of Ang-2 by nuclease-resistant RNA aptamers in rat corneal angigogenesis inhibited bFGF-mediated neo-vascularization, indicating that specific inhibitors of Ang-2 can act as anti-angiogenic agents (White 2003).

It is therefore possible that Ang-2, as an autocrine-acting antagonist, acts in a context-dependent manner as either an agonist or antagonist of Tie-2 signaling, and is dependent on the presence of pro-angiogenic activity (Fiedler 2004). In keeping with this observation, the angiopoietins have been associated with a number of different diseases, including vascular malformation, diabetes, pulmonary hypertension, arthritis, psoriasis, infertility and tumours.

These seemingly contradictory results strongly suggest that Ang-1 and Ang-2 are context-dependent modulators of angiogenesis and highlight the importance of targeting these factors in an appropriate spatial and temporal context (Iurlaro 2003; White 2003), in which the concentration, the duration of treatment and the accompanying factors together determine the resultant effect (Teichert-Kuliszewska 2001).
1.4.2.2 Ang-2 as a Homeostatic Contributor

The opposing effects of Ang-1 and Ang-2 support a model of constitutive Ang-1/Tie-2 interactions controlling vascular homeostasis as a default pathway, and Ang-2 acting as a dynamically regulated antagonizing cytokine (Fiedler 2004). This model is supported by the recent identification of Ang-2 as a component of Weibel-Palade bodies, co-localized with vWF (Fiedler 2004; Fiedler 2006). As mentioned previously, Weibel-Palade bodies are found in endothelial cells, and play a dual role in blood coagulation haemostasis and inflammation. The two major constituents of Weibel-Palade bodies are vWF, a multimeric protein involved in blood coagulation, and P-selectin, which binds passing leukocytes to the endothelium and subsequently facilitates transmigration across the endothelium. While Ang-2 and P-selectin storage in Weibel-Palade bodies was mutually exclusive (Fiedler 2004; Fiedler 2006), vWF was required for the trafficking of Ang-2 into the Weibel-Palade bodies (Fiedler 2004). Through the storage of these, and other, biologically active constituents, Weibel-Palade bodies provide a dynamic storage compartment whose contents can be regulated depending on the presence of inflammatory mediators in the vascular micro-environment (Rondaij 2006). The release of Weibel-Palade bodies is tightly regulated, and feedback mechanisms have been identified that prevent excessive release of bioactive components from this subcellular organelle (Rondaij 2006).

The few number of molecules identified in Weibel-Palade bodies, and the fact that they are all involved in controlling rapid vascular responses related to coagulation and inflammation (Fiedler 2004) suggests that Ang-2 also has important roles in these processes. As vascular morphogenetic programmes are primarily considered slow, transcriptionally-driven processes, the identification of Ang-2 as a stored, rapidly available molecule of endothelial cells therefore puts Ang-2 conceptually in a totally different biological context to its earlier roles, and raises a number of critical questions for the functional consequences of Weibel-Palade body-stored Ang-2 (Fiedler 2004; Fiedler 2006).

Experiments with endothelial cells showed that intracellular pools of Ang-2 can recover rapidly upon challenge, and that stored Ang-2 has a long half-life of more than 18 hours, and can be secreted within minutes of stimulation by PMA (phorbol-12 myristate 13-acetate), thrombin and histamine (Fiedler 2004). This identification of Ang-2 as a stored, rapidly available molecule in endothelial cells strongly suggests functions of the angiopoietin/Tie-2
system beyond the established roles during angiogenesis likely to be involved in rapid vascular homeostatic reactions such as inflammation and coagulation (Fiedler 2006).

1.4.2.3 Ang-2 and Inflammation

While Ang-1 has been reported to have anti-inflammatory properties, Ang-2 is thought to be pro-inflammatory (Fiedler 2004; Lemieux 2005; Roviezzo 2005; Fiedler 2006; Imhof 2006), and it has been proposed that it should be considered as an acute, pro-inflammatory mediator which may contribute to the initial steps of pathological angiogenesis (Lemieux 2005).

During inflammatory cell recruitment the entry of a pathogen induces the secretion of cytokines and chemokines, which activates neighbouring endothelial cells and attracts leukocytes. Leukocytes then move from blood to tissue by leukocyte rolling, activation, firm adhesion, and extravasation from the vasculature. This cascade occurs in a highly regulated manner, in which inflammatory cytokines such as TNF-α prompt upregulation of the adhesion molecules ICAM-1 and vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells. These adhesion molecules interact with leukocyte integrins to promote leukocyte adhesion and extravasation. Thus the expression of ICAM-1 and VCAM-1 during the early inflammatory response calibrates the outcome of the local immune reaction (Imhof 2006).

In a series of experiments, Fiedler et al showed that Ang-2 sensitizes endothelial cells to TNF-α and has a crucial role in the induction of inflammation (Fiedler 2006). Mice deficient in Ang-2 could not elicit an inflammatory response to immune challenge, while recombinant Ang-2 restored the inflammation defect (Fiedler 2006). Cellular experiments show that Ang-2 promotes adhesion of leukocytes to endothelial cells by sensitizing endothelial cells towards TNF-α and modulating TNF-α-induced expression of endothelial cell adhesion molecules ICAM-1 and VCAM-1. Together these findings identify Ang-2 as an autocrine regulator of endothelial cell inflammatory responses (Fiedler 2006). Ang-2 thereby acts as a switch of vascular responsiveness, exerting a permissive role for the activities of pro-inflammatory cytokines.

As mentioned previously, Tie-2 was initially thought to be an endothelial cell-specific receptor, however, recent investigations have shown its expression on a number of different cell types including endothelial precursor cells (Sato 1998; Ikeda 2004). Furthermore Tie-2 expression has also been noted on neutrophils (Lemieux 2005), which lead to the suggestion
that the Tie-2 receptor is maintained during the commitment of the hematopoietic progenitor cells. Angiopoietins were seen to modulate pro-inflammatory activities such as neutrophil adhesion onto endothelial cells and neutrophil migration (Lemieux 2005; Sturn 2005), suggesting that the angiopoietins might be instrumental in promoting acute recruitment of neutrophils, thus contributing to the facilitation of vascular remodeling and angiogenesis (Lemieux 2005).

Endothelial progenitor cells (De Palma 2005; Modarai 2005) are hematopoietic precursors which are proposed to potentially give rise to a number of different cell lineages, such as monocytes, endothelial cells and neutrophils (Schmeisser 2001). The identification of Tie-2 on endothelial progenitor cells (Sato 1995; Sato 1998) has led to the proposal that the Tie-2 receptor may be maintained by a number of cell types during differentiation. This includes the monocyte cell type, where current thinking is tending towards viewing monocytes as a collective term for a number of different sub-populations (including some expressing Tie-2) (Venneri 2007). These observations suggest that Ang-2 may be more involved in the regulation of the inflammatory response than previously anticipated.

1.4.2.4 Alternative Signaling pathways

An increasing number of reports suggest that the angiopoietins are able to interact directly with cells in the absence of Tie-2. In vitro studies show that human umbilical vein endothelial cells (HUVECs) adhere to both Ang-1 and Ang-2, as do fibroblasts, which do not express the Tie receptors, and show similar adhesion through integrin α5 (Carlson 2001). This suggests that angiopoietins can interact directly with members of the integrin family (Jones 2003). Similarly, cardiac and skeletal myocytes were seen to adhere to Ang-1 and Ang-2 via integrins (αv, α6, β1, β3), and their survival promoted by the interaction (Dallabrida 2005).

Ang-2 is upregulated in most tumours, and the balance between the angiopoietins shifts towards Ang-2 (Tait 2004). This observation has lead to the proposal of Ang-2 as a major player in the angiogenic switch in tumourigenic situations (Tait 2004), and that the production of Ang-2 is implicated in tumour progression, and that the over-expression of Ang-2 by cancer cells leads to enhanced tumour angiogenesis and growth of murine colon and gastric tumours in mice (Hu 2003). Ang-2 was seen to be related to tumour angiogenesis in gastric carcinomas in the absence of Tie-2 expression, and MMP-1, MMP-9 and uPA were strongly upregulated by Ang-2 in the presence of VEGF in the endothelial cells of these...
tumours *in vivo* (Etoh 2001). In human gliomas, Ang-2 was seen to induce the expression and activation of MMP2 (Hu 2003), and upregulation of Ang-2, MMP2, and MT1-MMP were associated with glioma invasion both *in vitro* and *in vivo* (Guo 2005), with the glioma cells responding directly to Ang-2 stimulus in the absence of Tie-2 expression. These results were confirmed by clinical investigations, where Ang-2, MMP2, MT1-MMP and lamininγ2 correlated with invasiveness of human gliomas (Guo 2005).

It is of importance to note that this responsiveness of glioma cells to Ang-2 occurs in the absence of detectable Tie-2 expression, through the integrin αvβ3 and the FAK signaling pathway (Hu 2006). It was suggested that the highly conserved COOH-terminal fibrinogen-like domain of the angiopoietins implies a functional association with the integrin receptor family, allowing Ang-2 to act as a potential substrate for the integrin receptor family in endothelial cells, fibroblasts and myocytes, as it not only enhances cell adhesion in both endothelial cells and Tie-2-deficient fibroblasts, but also triggers integrin-mediated intracellular signal transduction pathways in these cells.

Studies such as those mentioned above have begun to elucidate the many roles of Ang-2 aside from vessel destabilization and Tie-2 signaling. The ability of Ang-2 to bind directly to integrins on the cell surface suggests a wide range of alternative applications for this growth factor.

### 1.5 Synopsis of Experimental Design

In this study it was proposed that Ang-2 and PDGF-BB interact together with Ang-2 to upregulate monocyte fibrinolysis. Both Ang-2 and PDGF-BB, by virtue of their release mechanisms (Weibel-Palade bodies and degranulating platelets) would be present during the very early stages of wound healing. As this early wound healing is accompanied or preceded by an inflammatory infiltrate, consisting in large part of monocytes, it seemed possible that the growth factors would have some effect on the cells. Following extravasation from the blood vessels, monocytes encounter a fibrin clot formed during hemostasis. It therefore seemed possible that Ang-2 and PDGF-BB, in their action on monocytes, might affect the regulation of fibrinolytic elements in the cells.

Figure 3 is a basic flow diagram of the experimental design. All experiments utilized monocytes isolated from whole peripheral blood. Initial investigations into monocyte fibrin invasion were conducted using a novel fibrin invasion assay in which human peripheral
blood monocytes were seeded onto a fibrin clot suspended in medium. Cells were allowed to penetrate the clot, after which they were quantified, providing a correlative indication of the degree of fibrin invasion. This fibrin invasion assay was conducted under both serum and serum-free conditions, in the presence of both Ang-2 and PDGF-BB. Furthermore, it was repeated in the presence of broad-action inhibitors of fibrinolytic pathways.

Fibrinolysis was directly quantified using fibrin(ogen) immunoblotting, after which the expression elements of both the plasmin(ogen) and the MMP fibrinolytic systems was investigated on both the RNA and protein levels.

In order to determine the monocyte receptors through which Ang-2 and PDGF-BB were signaling, the expression of Tie-2 was investigated on both the RNA and protein level using flow cytometry. In the absence of signal, the expression of a number of integrins was examined on the monocytes under experimental conditions, and immunoprecipitation analyses of potential receptor complex was conducted. PDGFR-β expression was analysed on both an RNA and protein level, by RT-PCR, enzyme-linked immunosorbent assay (ELISA) and immunohistochemical analyses. Using various inhibitors, the possible signal transduction pathways for these receptors were also examined.

The effects of Ang-2 and PDGF-BB on fibrinolysis were also examined on other cell types known to be involved in wound healing, as well as on monocytes obtained from other species. Furthermore, the effect of Ang-2 and PDGF-BB on the proteolysis of other substrates was also investigated.

Finally, attempts were made to translate these in vitro findings into a viable in vivo model. The limitations of these studies will be discussed in the concluding chapter of the results.
Figure 3. Flow diagram of experimental design.
2

Materials and Methods

2.1 Development of an In Vitro Fibrin Invasion Assay

2.1.1 Isolation of Human Peripheral Blood Monocytes

This protocol was modified from the procedure outlined by Biddison in the Current Protocols in Cell Biology (Biddison 1998).

The blood donors used (as elaborated in 2.1.4) were healthy, non-smoking male adults under the age of 30, who had all abstained from any product containing aspirin for two weeks prior to the donation. The purpose of the research was fully explained to all potential donors, and donors were compensated for any inconvenience experienced.

In all donations, a qualified medical practitioner was employed to draw blood from the donors. 45 ml of blood was taken aseptically from the forearm of the donor, using a sterile 50ml syringe pre-prepared with 5 ml 0.1M trisodium citrate (BDH, Wadewille RSA, BB1024241) (Balasubramanian 2002). The trisodium citrate prevents Ca$^{2+}$-dependent activation of TF in whole blood, which leads to clotting. Once the blood had been drawn, all the following procedures were performed using sterile techniques in a Bio-Hazard Laminar Flow Hood. The blood was split between two 50ml tubes, and 25 ml room temperature phosphate buffered saline (PBS – see below) was added to each.

10 ml of Histopaque-1077 (Sigma, Steinheim, H8889) was pipetted into a 50 ml tube, and 20 ml of the blood/PBS mixture carefully layered on top using a 5 ml pipette. The layered blood was then spun at 2500 rpm for 20 minutes at 20 °C with the centrifuge brake off. This spin separated the contents of the tubes into visible layers, containing (from bottom to top) red blood cells, Histopaque-1077, mononuclear cells and plasma respectively. In all tubes, the top layer of plasma was aspirated off, and the mononuclear cell layer above the Histopaque collected using a 1ml Gilson pipette. The mononuclear cell layers from three separate Histopaque gradients were combined in a single fresh 50 ml tube, and PBS added up to the 50 ml mark. The tubes were then spun at 2200 rpm for 10 minutes at 20°C with the centrifuge brake on.
The resultant supernatant was discarded, and the pellet re-suspended in 50 ml of PBS. The tube was then spun at 1000 rpm for 15 minutes at 20°C. Once again the supernatant was discarded, and the pellet re-suspended in 50 ml of PBS. The tube was then spun at 1000 rpm for a further 15 minutes at 20°C. These washing steps removed the majority of platelets, which can cause cell clumping.

The resultant supernatant was discarded, and the pellet re-suspended in 20 ml of serum-containing RPMI-1640 (see below). This cell suspension was added to a 150 cm² Corning cell culture flask (Corning Inc, NY), which had been pre-coated with 5 µg/ml human plasma fibronectin (Roche, Mannheim, 1051407) for an hour at 37 °C. The flask was then left undisturbed in the incubator (37°C, 5 % CO₂) for 3 hours to allow for cell adhesion.

After incubation, the flask was rigorously washed twice with room temperature RPMI-1640 to remove non-adherent cells. 20 ml of room temperature PBSE (PBS with 5 mM EDTA (Sigma, Steinheim, ED355)) was then added to the adherent cells in the flask, and cell detachment monitored under a phase contrast microscope. Once the cells had detached, 20 ml of 4°C serum-containing RPMI-1640 was added to the flask, and the entire suspension transferred to a 50 ml tube.

The tube was spun at 1500 rpm for 10 minutes at 4°C. The resultant supernatant was discarded, and the cell pellet re-suspended in 1ml RPMI-1640. 20 µl of this cell suspension was diluted 1 in 25 with PBS, and then 1 in 2 with 0.4% Trypan blue (Fluka AG, Switzerland, 158951-85). The resultant suspension was loaded onto a hemacytometer, and cell concentration and viability was assessed.
2.1.2 Analysis of Monocyte Isolation Purity Using Flow Cytometry

The expression of the CD14 (BD, Franklin Lakes, 345 385) antibody on monocytes, was analysed as previously described (Herzenberg 2002) using direct immunofluorescent techniques. The monocytes were separated from peripheral blood using standard histopaque centrifugation (as detailed in 2.1.1) and resuspended in PBS with 0.5% bovine serum albumin (BSA, Sigma, Steinheim, A-4503).

In order to prevent nonspecific binding of the antibody, equal volumes of the cells and AB serum were incubated, at room temperature for ten minutes, prior to the addition of the antibody. Thereafter, 10 \( \mu \)l phycoerythrin (Pe)-conjugated CD14 (Becton Dickinson, 345-385) antibody was added. Pe-conjugated anti-IgG1 (Becton Dickinson) was used as a negative isotypic control. The cells were incubated at room temperature for thirty minutes. After the cells were washed thoroughly three times with excess PBS, analysis took place on a FACS Calibur flow cytometer (Becton Dickinson). A minimum of 2000 cells was counted with gating using forward and side scatter. The percentage positivity was assessed using single colour histograms.
All flow cytometric analyses were conducted with the assistance of Mrs Glenda Davison (Department of Haematology, UCT, Cape Town, South Africa) using flow cytometry machines belonging to the Department of Haematology.

2.1.3 Analysis of Monocyte Isolations Using Morphology

Monocytes were isolated as described in 2.1.1. After isolation, they were suspended in serum-containing RPMI-1640 growth medium, and seeded into 96 well plates (Corning, NY) at a concentration of 50000 cells per 100 μl. The cells were incubated at 37°C with 5% CO₂. Each day for 7 days, the wells were photographed using a 5x lens on a phase contrast microscope. Morphological changes in the cell population were monitored by an independent hematologist, Mrs Mona Bracher (CVRU, UCT, Cape Town).

2.1.4 Standardization of Blood Donation Criteria

Initial experiments were conducted with male and female blood donors of varying ages, with blood taken at different times of day in order to test the experimental protocol. All reported experiments use healthy, non-smoking male donors of between 20 and 30 years of age, who completed a medical questionnaire on their lifestyles, allergies, vitamin supplements, and general health (and medication). All blood was drawn at 11am and processed within 5 minutes of drawing.

2.1.5 Cryopreservation of Cells

In order to cryopreserve cells, monocytes were isolated as described in 2.1.1, and cultured for 24 hours in 75 cm² tissue culture flasks in serum-containing RPMI-1640 at 37 °C with 5% CO₂. The cells were then washed twice with PBS, and lifted using cell dissociation solution (Sigma, Steinheim, C1419).

The lifted cells were centrifuged at 1500 for 10 minutes and resuspended in 1 ml M199 cell culture medium (Gibco, 31100-027) with 10% fetal calf serum (Delta Bioproducts, RSA, 14-501 A1). A 50 μl aliquot was removed and added to an equal volume of Trypan Blue and viable cells counted with a haemocytometer. The cell concentration was then adjusted to 2 x 10⁶ cells per ml using M199 with 10% fetal calf serum (FCS. Delta Bioproducts, RSA, 14-501). The cell suspension was then placed on ice, and equal quantities of M199 \( /10\%\) FCS
/15% DMSO (Sigma, Steinheim, D-8779) was added to the cell suspension. This cell suspension was aliquoted into cryotubes (NUNC, Roskilde, 375418) in a polystyrene container and placed at -80°C overnight. The tubes were then transferred to a liquid nitrogen container (-196°C) where they were stored until use.

2.1.6 Preparation of Fibrinogen

Plasminogen-depleted human plasma fibrinogen (Fluka Biochemika 46313), 700 mg /20ml PBS was dialysed twice against 2 litres of PBS for 1.5 hours, and once against 1 litre Heps buffered saline (HBS – see below). The dialysed fibrinogen solution was centrifuged at 1000xg for 10 mins and filtered successively through 0.8 µm, 0.45 µm, and 0.22 µm low protein binding Millipore filters (Millipore SLGV025LS). Concentration was calculated by absorbance at A280 using an extinction coefficient of 1.55.

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBS buffer</td>
<td>10 mM HEPES</td>
<td>Sigma, Steinheim, H-3375</td>
</tr>
<tr>
<td></td>
<td>140 mM NaCl</td>
<td>Sigma, Steinheim, S-7653</td>
</tr>
<tr>
<td></td>
<td>5 mM CaCl₂</td>
<td>ACE, Reuven, 7383</td>
</tr>
<tr>
<td></td>
<td>Filtered with a 0.22 µm low protein binding filter</td>
<td>Milipore, Bedford, SCGVT01RE</td>
</tr>
</tbody>
</table>

2.1.7 Characterisation of Fibrin Gels Using Scanning Electron Microscopy

Fibrin gels were cast in a 96 well Teflon well plate made for the purpose by Mr Bruno Orlandi. 2.5µl of bovine thrombin stock (2U/10 µl, Sigma, Steinheim, T7513) was added to 397.5 µl of HBS, and 28 µl of this mixture was pipetted into each well with 7 µl of purified human plasma fibrinogen stock (18.9 mg/ml, Fluka, Switzerland, 46313). After the fibrin had set, the gels were carefully removed from the wells, and fixed using 2% gluteraldehyde in PBS for 10 minutes, after which it was rinsed twice in PBS. The gels were then dehydrated by successive 10-minute incubations in deionized water, 50% ethanol, 70% ethanol, 80% ethanol, 90% ethanol and 100% ethanol. The dehydrated fibrin gel was then critical point dried (CPD) with liquid CO₂ using a Polaron CPD 7501 (Quorum Technologies, Newhaven, UK). Following CPD the samples were sputter coated with gold palladium using a Polaron SC7640 (Quorum Technologies, Newhaven, UK).

These samples were viewed at 25 kV using a JSM 5200 scanning electron microscope (Jeol, Tokyo, Japan), and the images captured and analysed using the Orion V5.20 image analysis
2.1.8 Analysis of Fibrin Gel Degradation

While the fibrinogen prepared in 2.1.6 was plasminogen-depleted, it was not possible to guarantee its complete absence. It was therefore decided that the experimental conditions should be assayed for background fibrin degradation.

One of the major products of fibrin degradation are D-dimers (Bini 1996; Bini 1999). A D-dimer ELISA produced by Technoclone (Technoclone, Vienna, 2599006) was utilized to provide a basic overview of the levels of fibrin degradation in both serum-containing and serum-free RPMI-1640. Fibrin gels were cast in a 96 well plate. 2.5 μl of bovine thrombin stock (2U/10 μl, Sigma, Steinheim, T7513) was added to 397.5 μl of HBS, and 28 μl of this mixture was pipetted into each well with 7 μl of purified human plasma fibrinogen stock (18.9 mg/mL, Fluka, Switzerland, 46313). The solution was then thoroughly mixed, and left to polymerise for an hour at room temperature.

To half the wells, serum-free RPMI-1640 (see below) was added, while to the other half serum-containing RPMI-1640. Monocytes isolated as described in 2.1.1 were seeded on top of the fibrin in half of the wells for each serum-type. The wells were incubated for 24 hours at 37°C with 5 % CO2 before the medium was drawn off.

The ELISA was conducted according to the manufacturer's specification. Briefly, ELISA wells were thoroughly washed with washing buffer before the sample was added. Once added, the samples were incubated for 37°C for an hour. Following this, the samples were then aspirated off, and the wells washed again with three separate washes. The conjugation solution was then added and the wells incubated again for another hour at 37°C. The wells were then washed as before, and incubated with substrate solution for 10 minutes. The colour development was stopped using 1M sulfuric acid, and measured using a microplate reader (Biorad, California, Benchmark) at 450 nm, with a reference wavelength of 690 nm.
Material | Preparation | Supplier / Product no.
--- | --- | ---
Serum-free RPMI-1640 | RPMI-1640 medium with 2g/l NaHCO₃ added. Filtered with 0.22 μm low protein binding filter 0.1% bovine serum albumin (BSA) 1% ITS liquid medium supplement | Sigma, Steinheim, R-6504 Sigma, Steinheim S-5761 Milipore, Bedford, SCGVT01RE Sigma, Steinheim, A-4503 Sigma, Steinheim, I-3146

2.1.9 Transwell Fibrin Invasion Assay

![Transwell Fibrin Invasion Assay](image)

This assay was based on a model used in previous monocyte migration studies (Ciano 1986). It makes use of Transwells with a larger pore size (8 μm), with a fibrin layer added to the upper side of the microporous membrane.

Upregulated invasion and fibrinolysis would shorten the time interval before which the cells would come into contact with the exposed microporous membrane. The increased pore size of these membranes ensures that movement of cells through the membrane would be relatively uninhibited. Increased invasion into the fibrin clot can therefore be directly quantified by, and reflected in, an increased number of cells in the lower compartment.

The fibrin gel on the upper side of the microporous membrane was prepared in the following way. Bovine thrombin stock (2U/10 μl, Sigma, Steinheim, T7513) was diluted 1/160 in HBS buffer. 28 μl of this mixture was pipetted onto the microporous membrane. Purified human plasma fibrinogen stock (18.9 mg/ml, Fluka, Switzerland, 46313) was then carefully mixed into the thrombin mixture to form a resultant fibrinogen concentration of 3mg/ml. The mixture was left to polymerise for an hour at room temperature.
The fibrin-coated membranes were seeded with monocytes isolated as described in 2.1.1. The cells were then incubated in serum-containing RPMI-1640 at 37°C with 5% CO₂ and the migration of the cells was estimated by monitoring the appearance of cells in the bottom well. After a 19-hour incubation, the cells in the lower compartments were fixed and analysed in the following manner.

After the removal of the Transwell insert, cells in the lower compartment were fixed overnight at 4°C with 4% formaldehyde. The wells were then analysed using a phase contrast microscope with a 2.5x objective (Leica DM IRBE, Solms, Germany). Once an image of the cells in the bottom well was captured using the Q-Win software associated with the microscope, the number of cells in the image was determined using the “count” function of the software. Both the number of cells, and the total area of the cells were noted for each well. The “edit” function of the software allowed the researcher to manually exclude cellular debris prior to the count. For each study, the contrast was set before the analysis, and not altered throughout the analysis.

2.1.10 DAPI Analysis of Invading Cells

Cells present in the bottom compartment after incubation of the fibrin invasion assay (2.1.9) were subjected to a DAPI nuclear stain. Cells were washed twice with PBS, and DAPI (Promega, Madison, P5521) was added at a concentration of 1/10000 in PBS. The cells were incubated for an hour at room temperature, after which the stain was aspirated off, and the cells washed thoroughly three times with PBS. The DAPI stain was investigated using a fluorescent microscope (Olympus) using an absorption maximum of 358 nm and an emission maximum of 461 nm.

2.2 Analysis of Monocyte Fibrin Invasion in Response to Ang-2 and PDGF-BB

2.2.1 Serum-Containing Fibrin Invasion Assays

The fibrin invasion assay described above (2.1.9) was modified by the addition of carrier-free Ang-2 (R&D Systems, Minneapolis, 623-AN-025/CF) to the serum-containing RPMI-1640 in the lower compartment. For the dilution curve, Ang-2 was added to triplicate wells at dilutions of 100 ng/ml, 250 ng/ml, 500 ng/ml and 1 μg/ml. In all further experiments Ang-2
Table 2. Material Preparation and Supplier/ Product no.

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/ Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum-free RPMI-1640</td>
<td>RPMI-1640 medium with 2g/l NaHCO₃ added. Filtred with 0.22 µm low protein binding filter. 0.1% bovine serum albumin (BSA) 1% HBS liquid medium supplement pH 7.4</td>
<td>Sigma, Steinhein: P 4501 Sigma, Steinhein: S 8571 Milipore, Bedford, ECGT319RE Sigma, Steinhein: A-4208 Sigma, Steinhein: 1-8146</td>
</tr>
</tbody>
</table>

2.1.9 Transwell Fibrin Invasion Assay

This assay was based on a model used in previous monocyte migration studies (Ciano 1988). It makes use of Transwells with a larger pore size (8 µm), with a fibrin layer added to the upper side of the microporous membrane.

Upregulated invasion and fibrinolysis would shorten the time interval before which the cells would come into contact with the exposed microporous membrane. The increased pore size of these membranes ensures that movement of cells through the membrane would be relatively uninhibited. Increased invasion into the fibrin clot can therefore be directly quantified by, and reflected in, an increased number of cells in the lower compartment.

The fibrin gel on the upper side of the microporous membrane was prepared in the following way. Bovine thrombin stock (2U/10 µl, Sigma, Steinhein, T7513) was diluted 1/100 in HBS buffer. 28 µl of this mixture was pipetted onto the microporous membrane. Purified human plasma fibrinogen stock (18.9 mg/ml, Fluka, Switzerland, 46313) was then carefully mixed into the thrombin mixture to form a resultant fibrinogen concentration of 3mg/ml. The mixture was left to polymerise for an hour at room temperature.
the membrane in both the treated wells and the control wells can provide an indication as to the chemotactic properties of the growth factor.

Transwell permeable support chambers with 5 μm pores were purchased from Corning (Corning, NY, 3421). The upper compartment of each Transwell was seeded with monocytes (isolated as detailed in 2.1.1), at a concentration of 50 000 cells in 100 μl serum-containing RPMI-1640.

500 μl of serum-containing RPMI-1640 was placed in the lower compartment, to which Ang-2 (R & D, Minneapolis, 623-AN-025/CF) or PDGF-BB (PeproTech Inc, Rocky Hill, 100-14) was added at concentrations of 500 ng/ml or 20 ng/ml respectively.

The cells were then incubated at 37°C with 5% CO₂ for 5 hours, as determined by the literature (Emofsson 1996). After incubation, the Transwell inserts were transferred to sterile 24 well plate into wells containing containing 4% formaldehyde (Merk, Darmstadt, AC004003.2.5), and incubated overnight at 4°C. The inserts were then incubated for 5 minutes in Mayers haematoxylin (see below) washed for 5 minutes under running water, and then rinsed twice in 100 % ethanol (Merk, Darmstadt, AB000983.2.5). Before allowing the membrane to dry, the cells and excess fibrin on the upper side of the membrane were wiped off using a cotton-wool bud soaked in PBS.

After allowing the excess ethanol to evaporate from the membrane, each membrane was excised from the supporting upper compartment using a scalpel. The membranes were then mounted on micro slides (Marienfeld, Lauda-Koningshofen, 75x25mm), using aqueous mounting medium containing 15 mM NaCl (Dako, Glostrup, S3025).

Each membrane was analysed by the researcher as well as an independent observer on a light microscope (Olympus) in the following manner: 5 representative fields were chosen from the whole membrane at a magnification of 20x. The number of cells in each field were counted and recorded.
### Material

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2g Haematoxylin</td>
<td>Merck, Darmstadt, 15938</td>
</tr>
<tr>
<td>50g Potassium Alum</td>
<td>Saarchem, Krugersdorp, RSA, 5823480</td>
</tr>
<tr>
<td>0.2g Sodium Iodate</td>
<td>Saarchem, Krugersdorp, RSA, 1605020</td>
</tr>
<tr>
<td>Dissolve in 1L distilled water using gentle heat</td>
<td>Saarchem, Krugersdorp, RSA, 1591500</td>
</tr>
<tr>
<td>1g Citric Acid</td>
<td></td>
</tr>
<tr>
<td>50g Chloral hydrate</td>
<td></td>
</tr>
<tr>
<td>Add and dissolve well. Boil 5 mins Cool filter with 0.22 µm low protein binding filter</td>
<td>Milipore, Bedford, SCGV01RE</td>
</tr>
</tbody>
</table>

### 2.2.3 Cell Proliferation ELISA

In order to ensure that any alteration in cell numbers seen in the cell invasion assays was not an artifact of increased cell proliferation, the effects of Ang-2 on monocyte proliferation in a serum-containing environment were measured using a Bromo-deoxy-Uridine (BrdU) colorimetric cell proliferation ELISA (Roche, Mannheim, 1 647 229).

Monocytes isolated as described in 2.1.1 were seeded into serum-containing RPMI-1640 in a 96 well plate (Corning Inc, NY) at a concentration of 50000 cells per well. The cells were allowed to settle for 2 hours before Ang-2 was added to the experiment wells at a concentration of 500 ng/ml.

Human saphenous vein endothelial cells (HSVEC) stimulated with bFGF were chosen as a positive control (personal communication, Dr Neil Davies, CVRU, UCT, South Africa). Human saphenous vein was obtained from discard by the Cardio-Thoracic Surgery Unit at Groote Schuur Hospital, Cape Town, South Africa. HSVEC were isolated from the fresh tissue by Mrs Ronnett Seldon (CVRU, UCT, Cape Town).

Cells stocks were stored in 7.5% dimethyl sulphoxide (DMSO -Sigma, Steinheim, D-8779) and 10% heat inactivated human pooled serum (Delta Bioproducts, RSA) in M199 media (Sigma, Steinheim, M5017), and maintained as frozen stock in liquid nitrogen at -196°C.
For use, cells were thawed at 37°C and seeded directly into 150 cm² culture flasks (Corning Inc, NY) containing 30 ml of the endothelial growth medium (see below) containing 10% fetal calf serum (FCS), and incubated at 37°C with 5% carbon dioxide for 24 hours. Following the incubation period, the medium was replaced with fresh growth medium with 10% FCS, and the flasks returned to the incubator. Cells were used at passage 3 for experimental purposes. HSVEC were seeded into endothelial growth medium (see below), and (additional) bFGF (1 μg/ml) added to the wells. All wells were incubated at 37 °C with 5% CO₂ for 24 hours.

After the incubation period, the BrdU proliferation marker was added to the wells at a final concentration of 10 μM. The wells were then incubated overnight, and assayed according to the manufacturer's specifications in the morning. Briefly, following the incubation, the labeling medium was removed by aspiration and 200 μl of FixDenat was added to the cells. After a 30-minute incubation, this was removed by aspiration, and the anti-BrdU-POD antibody added at a working concentration of 1/100. After 90 minutes of incubation, the cells were washed thoroughly with three separate washes of washing solution.

After the final wash, substrate solution was added to the wells, and the cells incubated at room temperature for 10 minutes to allow for colour development. Colour development was stopped with 1M sulfuric acid. Absorbance was measured using a microplate reader (Biorad, California, Benchmark) at a wavelength of 450 nm, with a reference wavelength of 690 nm.

Control wells for the assaying procedure were set up as described below:

<table>
<thead>
<tr>
<th>Well contents</th>
<th>Blank</th>
<th>Background control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture medium</td>
<td>100 μl</td>
<td>-</td>
</tr>
<tr>
<td>Cells</td>
<td>-</td>
<td>100 μl</td>
</tr>
<tr>
<td>BrdU (100 μM)</td>
<td>10 μl</td>
<td>-</td>
</tr>
<tr>
<td>Anti-BrdU-POD</td>
<td>100 μl</td>
<td>100 μl</td>
</tr>
</tbody>
</table>
Endothelial growth medium

MCDB medium 131 with 1.18g/l NaHCO₃ added
Filtered with 0.22 µm low protein binding filter
1% penicillin / streptomycin
2 mM L-glutamate
10 ng/ml epidermal growth factor
5 ng/ml basic·fibroblast growth factor
1 g/ml Hydrocortisone
pH to 7.4

2.2.4 LIVE/DEAD® Viability/Cytotoxicity kit

The LIVE/DEAD® Viability/Cytotoxicity Kit (Molecular Probes, Eugene, L-3224) was obtained in order to check the viability of cells after various treatments with inhibitors. The kit contains two different solutions – calcein AM and ethidium homodimer (EthD-1). Live cells are distinguished by the presence of ubiquitous intracellular esterase activity, which can be determined by the enzymatic conversion of the virtually non-fluorescent cell permeant, calcein AM to the intensely fluorescent ccalcine. This fluorescent signal is retained by living cells, producing intense uniform green fluorescence in live cells. EthD-1 enters cells with damaged membranes and undergoes a 40-fold enhancement of fluorescence upon binding to nucleic acids, thereby producing a bright red fluorescence in dead cells.

The assay was conducted according to the manufacturer’s specifications. Briefly, monocytes were seeded into a 96 well plate and cultured in serum-containing RPMI-1640 (2.1.1) in the presence of various inhibitors such as aprotinin (Bayer, Germany, 01057001, 50 ng/ml); GM6001 (Chemicon International, Temecula, CC-1000. 0.05 µg/ml); amelioride (Sigma, Steinheim, A74410-IG, 50 µM); cycloheximide (Sigma, Steinheim, C-1988); wortmannin (Sigma, Steinheim, W-1628, 0.1 µM); SB239063 (Sigma, Steinheim, S0569, 100 nM) and apigenin (Sigma, Steinheim, A3145, 10 µM). Following a 24-hour incubation at 37°C with 5% CO₂, cultured cells were rinsed thoroughly in sterile PBS. The calcein AM (1 µM) and EthD-1 (2 µM) were added and the cells incubated at 37°C for 30 minutes.

Following this incubation, fluorescence in experimental and control cell samples were measured using the appropriate excitation and emission filters (calcein: 494/517 nm, EthD-1: 528/617 nm) using a fluorimeter.
Controls were prepared as follows:

<table>
<thead>
<tr>
<th>Description of control</th>
<th>Assignation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluorescence at 634 nm in the experimental cell sample labeled with calcein AM and EthD-1</td>
<td>F(645)max</td>
</tr>
<tr>
<td>Fluorescence at 530 nm in the experimental cell samples labeled with calcein AM and EthD-1</td>
<td>F(530)max</td>
</tr>
<tr>
<td>Fluorescence at 645 nm in a sample where all the cells are dead, labeled with EthD-1 only</td>
<td>F(645)max</td>
</tr>
<tr>
<td>Fluorescence at 645 nm in a sample where all the cells are dead, labeled with calcein AM only</td>
<td>F(645)min</td>
</tr>
<tr>
<td>Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with EthD-1 only</td>
<td>F(530)min</td>
</tr>
<tr>
<td>Fluorescence at 530 nm in a sample where all (or nearly all) cells are alive, labeled with calcein AM only</td>
<td>F(530)max</td>
</tr>
<tr>
<td>Fluorescence at 530 nm of a cell-free sample with dye added</td>
<td>F(530)o</td>
</tr>
<tr>
<td>Fluorescence at 645 nm of a cell-free sample with dye added</td>
<td>F(645)o</td>
</tr>
</tbody>
</table>

The percentage live and dead cells can be calculated from the fluorescence readings determined by the following equations:

\[
\% \text{ live cells} = \frac{F(530)_{\text{max}} - F(530)_{\text{min}}}{F(530)_{\text{max}} - F(530)_{\text{min}}} \times 100\% \\
\% \text{ dead cells} = \frac{F(645)_{\text{max}} - F(445)_{\text{min}}}{F(645)_{\text{max}}} \times 100\%
\]

2.2.5 Analysis of Growth Factors in Serum

Aliquots of serum-containing RPMI-1640 were analysed using two different commercially available ELISA kits. VEGF and PDGF-BB levels were determined using ELISA kits from R&D (R&D, Minneapolis, DY293B and DY220).

Both ELISAs were conducted according to manufacturers' specifications. Briefly, ELISA plates were incubated with working solutions of capture antibody in wells overnight at room temperature according to manufacturer's specifications. Following this incubation, the wells were washed 4 times, and block buffer added for an hour, also at room temperature. This was then aspirated off, and the wells washed as before.

The samples were then added to the prepared plate, and incubated at room temperature for 2 hours. They were then aspirated off, the wells washed as before, and the detection body was
added to the wells. After a 2-hour incubation, the wells were washed and aliquots of avidin peroxidase added. The wells were incubated for 30 minutes at room temperature, after which they were washed thoroughly. Substrate solution was then added to each well, and the wells incubated at room temperature to allow for colour development.

The plates were read using a microplate reader (Biorad, California, Benchmark) at 405 nm wavelength, with correction set at 650 nm.

2.2.6 Serum-Free Fibrin Invasion Assay

Monocytes were isolated as described in 2.1.1. After lifting the cells from the flask with PBSE, cells were resuspended in RPMI-1640. Transwells were coated with fibrin as described in 2.1.9, however coated wells were placed in compartments containing serum-free RPMI. These Transwells were seeded with monocytes at a concentration of 50000 cells/well. The cells were allowed to settle for 2 hours, after which the Transwells were treated as follows:

PDGF-BB (PeproTech Inc., Rocky Hill, 100-14B) was used at 15 ng/ml (Ernofsson 1996), while Ang-2 was used at 500 ng/ml. The Transwells were incubated for 19 hours, after which the cells in the lower compartments were fixed with 4% formaldehyde, and analysed as described in 2.1.9.

The serum-free invasion assay was also repeated with the addition of goat polyclonal antibody to human uPA receptor (Abcam, Cambridge, ab3129. 4 μg/ml) and mouse monoclonal to human integrin β2 (Chemicon, Temecula, CA, MAB1927. 3 μg/ml) to the fibrin gel, as well as to the top and bottom compartments.
2.3 Production and Analysis of Monocyte Conditioned Medium and Cell Lysate

2.3.1 Production of Monocyte Conditioned Medium and Cell Lysate

2.3.1.1 Production of Conditioned Medium

Monocytes were isolated as described in 2.1.1. Cells were seeded into 96 well plates (Nunc, Roskilde) at a concentration of 50000 cells/well in serum free RPMI medium (2.1.8). The cells were allowed to settle for an hour, after which Ang-2 and PDGF-BB were added to the experimental wells at a concentration of 500 ng/ml and 20 ng/ml respectively. The wells were then incubated at 37°C with 5% CO₂. In certain experiments, the inhibitors aprotinin (50 ng/ml), GM6001 (6 μg/ml) and anti-uPAR (4 μg/ml) were also added to the culture medium. After 15 hours of incubation, the conditioned medium from triplicate wells was drawn off, and stored at -20°C until use.

2.3.1.2 Extended Time Analysis of Conditioned Medium

Monocytes were isolated as described in 2.1.1. They were seeded into 96 well plates containing 100 μl of serum-containing RPMI-1640 at a concentration of 50000 cells per well. The cells were allowed to settle for 2 hours. After two hours, the medium was aspirated off 3 experiment wells, and 3 control wells. In these wells, the complete medium was replaced with serum-free RPMI-1640. Ang-2 and PDGF-BB were added to the experiment wells at 500 ng/ml and 20 ng/ml respectively, and the wells incubated at 37°C with 5 % CO₂. Conditioned medium was drawn off after 24 hours and stored at -20°C. This incubation in serum free conditions was repeated with triplicate wells on successive days from day 1 to day 4.

2.3.1.3 FDP-Containing Conditioned Medium

96 well plates were coated with a fibrin gel layer in the following way. 2.5 μl of bovine thrombin stock (2U/10 μl) was added to 397.5 μl of HBS buffer (2.1.6). 28 μl of this mixture was pipetted into the well. 7 μl of human plasma fibrinogen stock (18.9 mg/ml) was then
carefully mixed into the thrombin mixture. The mixture was left to polymerise for an hour at room temperature.

Wells were seeded with monocytes isolated as described above (2.3.1.1), and Ang-2 and PDGF-BB added to the wells as described before. The wells were then incubated at 37°C with 5% CO₂. At 15 hours, the conditioned medium from triplicate wells was drawn off, and stored at -20°C until use.

2.3.1.4 Cell Lysates

12 well plates (Nunc, Roskilde) were seeded with monocytes (isolated 2.1.1) at a concentration of 550 000 cells per well. Ang-2 and PDGF-BB were added to the serum-free RPMI-1640 at 500 ng/ml and 20 ng/ml respectively. At 15 hours conditioned medium was aspirated off triplicate wells, and the wells washed twice with PBS. 50 µl RIPA lysis buffer (see below, (Maquoi 1998)) was then added to each well, the contents of each well scraped with a rubber policeman and the wells incubated for 1 hour at 4°C. The fluid was then drawn off, and the process repeated with another 50 µl of RIPA lysis buffer. The lysates from both incubations were pooled and stored at -80°C.

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>RIPA lysis buffer</td>
<td>50 mM Tris-HCl (pH 7.4)</td>
<td>Sigma, Steinheim, T-6066</td>
</tr>
<tr>
<td>(Pichiule 2004)</td>
<td>150 mM NaCl</td>
<td>Sigma, Steinheim, S-7653</td>
</tr>
<tr>
<td></td>
<td>1% Nonidet P40</td>
<td>Sigma, Steinheim, I-3021</td>
</tr>
<tr>
<td></td>
<td>1% Triton X-100</td>
<td>Sigma, Steinheim, T-8532</td>
</tr>
<tr>
<td></td>
<td>1% Na-deoxycholate</td>
<td>Sigma, Steinheim, D-8750</td>
</tr>
<tr>
<td></td>
<td>0.1% SDS</td>
<td>Sigma, Steinheim, L-3771</td>
</tr>
<tr>
<td></td>
<td>5 mM Iodoacetamide</td>
<td>Sigma, Steinheim, I-1149</td>
</tr>
<tr>
<td></td>
<td>2 mM Phenylmethylsulfonyl fluoride</td>
<td>Sigma, Steinheim, P-7626</td>
</tr>
</tbody>
</table>
2.3.2 General Immunoblotting Technique

2.3.2.1 Reducing SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

<table>
<thead>
<tr>
<th>Blocking for</th>
<th>Samples used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-2</td>
<td>As detailed in 2.2.2</td>
</tr>
<tr>
<td>β-actin</td>
<td>As detailed in 2.3.1.4</td>
</tr>
<tr>
<td>FDP</td>
<td>As detailed in 2.3.1.3</td>
</tr>
<tr>
<td>Integrin β1</td>
<td>As detailed in 2.3.1.4</td>
</tr>
<tr>
<td>MMP3</td>
<td>As detailed in 2.3.1.1</td>
</tr>
<tr>
<td>MMP7</td>
<td>As detailed in 2.3.1.1</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>As detailed in 2.3.1.4</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>As detailed in 2.3.1.4</td>
</tr>
<tr>
<td>uPAR</td>
<td>As detailed in 2.3.1.4</td>
</tr>
</tbody>
</table>

Samples were diluted with the sample buffer (see below) in a 4:1 ratio, and heated at 90°C for 4 minutes to further assist denaturation.

10% polyacrylamide gels containing 0.01% SDS were prepared using the Biorad Miniprotean II vertical electrophoresis system (Biorad, California, 165-2940). The composition of the stacking and separating gels are detailed below.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Separating (10%) gel</th>
<th>Stacking (4%) gel</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris Buffers</td>
<td>2.5 ml (1.5M, pH 8.8)</td>
<td>1.65 ml (0.5M, pH 8.8)</td>
</tr>
<tr>
<td>Water</td>
<td>4.1 ml</td>
<td>3.35 ml</td>
</tr>
<tr>
<td>10% w/v SDS</td>
<td>100 µl</td>
<td>65 µl</td>
</tr>
<tr>
<td>Acrylamide /</td>
<td>3.3 ml</td>
<td>1 ml</td>
</tr>
<tr>
<td>bis Acrylamide (30 %)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium Persulphate</td>
<td>44 µl</td>
<td>88 µl</td>
</tr>
<tr>
<td>(50 mg/ml)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>9 µl</td>
<td>15 µl</td>
</tr>
</tbody>
</table>

Table 6. Detailing the composition of the 10% polyacrylamide gel

In all gels, 5 µl of pre-stained SDS-PAGE standards (Biorad, California, 161-0374), based on the Laemmli method, was loaded to allow for the quantification of molecular weights. The gels were run at a constant current of 25mA.
<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris buffer</td>
<td>Tris</td>
<td>Sigma, Steinheim, T-6066</td>
</tr>
<tr>
<td>Acrylamide /</td>
<td>29% (w/v) acrylamide (ultrapure)</td>
<td>Sigma, Steinheim, A-3553</td>
</tr>
<tr>
<td>bisacrylamide</td>
<td>I% (w/v) N,N'-methylene-bis-Acrylamide</td>
<td>Sigma, Steinheim, M-7279</td>
</tr>
<tr>
<td>(30%)</td>
<td>0.22 μm filtered</td>
<td>Millipore, Bedford, SCGVT01RE</td>
</tr>
<tr>
<td>Sample buffer</td>
<td>200 mM Tris base</td>
<td>Sigma, Steinheim, T-6066</td>
</tr>
<tr>
<td>(4x)</td>
<td>3% (w/v) SDS</td>
<td>Sigma, Steinheim, L3771-100G</td>
</tr>
<tr>
<td></td>
<td>0.1% (w/v) Bromophenol blue</td>
<td>Sigma, Steinheim, B-8026</td>
</tr>
<tr>
<td></td>
<td>40% (v/v) glycerol</td>
<td>Sigma, Steinheim, G-2025</td>
</tr>
<tr>
<td></td>
<td>pH 6.8</td>
<td>Sigma, Steinheim, M-7154</td>
</tr>
<tr>
<td></td>
<td>2.5% (v/v) 2-mercaptoethanol added</td>
<td>Sigma, Steinheim, M-7154</td>
</tr>
<tr>
<td></td>
<td>immediately before use</td>
<td></td>
</tr>
<tr>
<td>Tank buffer</td>
<td>0.3% (w/v) Tris base</td>
<td>Sigma, Steinheim, T-6066</td>
</tr>
<tr>
<td></td>
<td>1.4% (w/v) glycine</td>
<td>BDH, Wadeville RSA, BB101196N</td>
</tr>
<tr>
<td></td>
<td>0.1% SDS</td>
<td>Sigma, Steinheim, L3771-100G</td>
</tr>
</tbody>
</table>

### 2.3.2.2 Immunoblotting

Semi-dry transfer of proteins from gel to nitrocellulose membrane were executed using the Trans-blot SD Semi-Dry Transfer system (Bio-Rad, California). Prior to transfer, the blotting paper and nitrocellulose membrane were soaked for 10 minutes in transfer buffer (see below). The transfer-stack was assembled in the following way: 3 x blotting paper (Sigma, Steinheim); Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Little Chalfont, RPN303D) cut to fit the gel; the polyacrylamide gel containing the separated proteins (with stacking gel and wells removed); 3 x blotting paper. The stack was placed on the machine stage with the membrane towards the positive electrode.

The proteins in the polyacrylamide gel were electrophoretically transferred to the nitrocellulose membrane at 15V for 45 minutes. Following the transfer, membranes were dried overnight at room temperature.

Dried membranes were blocked with 50 ml TBS-T (see below) containing 5% non-fat milk powder (Parma lat, RSA) for 1 hour at room temperature with agitation. Following the incubation, three washes of 10 minutes each in 10 ml TBS-T were performed.
Table 7. Detailing the primary antibodies used during immunoblotting

The primary antibody was diluted in TBS-T containing 5% non-fat milk powder. The final volume antibody solution was calculated for 100 μl per cm² of membrane. All blots were incubated with the primary antibody for an hour at room temperature with agitation. Following the incubation, the membrane was washed twice for 10 minutes each in TBS-T.

Table 8. Detailing the secondary antibodies used in immunoblotting

The dilution volumes of the secondary antibodies were calculated as for the primary ones (see above) and all antibodies diluted in TBS-T. All blots were incubated with the secondary
antibody for an hour at room temperature with agitation. Following the incubation, the membrane was washed twice for 10 minutes and once for 5 minutes in TBS-T.

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfer buffer</td>
<td>25 mM Tris base</td>
<td>Sigma, Steinheim, T-6066</td>
</tr>
<tr>
<td></td>
<td>193 mM glycine</td>
<td>BDH, Wadeville, RSA, BB101196N</td>
</tr>
<tr>
<td></td>
<td>20 % methanol</td>
<td>Illovo, Johannesburg, RSA</td>
</tr>
<tr>
<td>TBS-T</td>
<td>20 mM Tris base</td>
<td>Sigma, Steinheim, T-6066</td>
</tr>
<tr>
<td></td>
<td>137 mM sodium chloride</td>
<td>Sigma, Steinheim, S-7653</td>
</tr>
<tr>
<td></td>
<td>0.1% (v/v) Tween-20</td>
<td>Sigma, Steinheim, P-7949</td>
</tr>
<tr>
<td></td>
<td>pH 7.6</td>
<td></td>
</tr>
</tbody>
</table>

2.3.2.3 Detection and Analysis

In order to visualize the membrane, equal aliquots of ECL detection solutions 1 and 2 (Amersham Pharmacia, Little Chalfont, RPN 2106) were mixed and pipetted over the membrane. The final volume of the detection solutions was calculated to be 0.125 ml per square centimeter of membrane (manufacturer's specifications). The membrane was incubated for 1 minute with the detection solutions before they were poured off.

The membrane was then wrapped in cling-film and placed onto X-ray Hyperfilm (Amersham, Little Chalfont). After a detection time of 1 - 10 minutes, the film was developed using Structurix developer and fixer (Agfa, Mortsel, G128 and G333c). The film was then rinsed thoroughly and air-dried.

The developed x-ray films were scanned using a Flat-Bed scanner (Umax Astra 1220S) using a transparent media adapter, interfaced to a Macintosh computer. For scanning Adobe Photoshop was used (Adobe Systems Inc, Mountainview, California) in gray-scale mode with scanner spatial resolution at 100 dpi. The image was saved as a TIFF file for machintosh.

The images were analyzed using of the NIH (National Institute of Health) 1.62 densometric analysis software. Lanes containing the bands of interest were marked for analysis and the optical density (number of pixels) of each band was recorded, and a curve plotted. The height of the curve is the mean of the OD of a given row of pixels in the marked lanes. Using the line drawing tool, base lines were drawn beneath each peak so that the areas beneath the curve were sealed for measurement. The area of each peak was measured using the wand.
tool, and the measured areas analysed using the uncalibrated OD option. The values were automatically annotated to the graph.

2.3.3 Zymography

Zymography is an electrophoretic technique used to identify proteolytic activity in enzymes separated in standard Laemmli SDS polyacrylamide gels co-polymerised with a protein substrate (gelatin) under non-reducing conditions.

After the removal of SDS from the gel, the denatured MMPs are able to refold themselves and digest the substrate (gelatin). Once the gel is stained with Coomassie Blue, the digested area is visible as a clear band, the size of which is proportional to enzyme concentration and activity in the sample.

2.3.3.1 Non-Reducing Polyacrylamide Electrophoresis with Gelatin or Caesin

Polyacrylamide gels with a 10% separating gel, and a 4% stacking gel were prepared as in 2.3.2.1, however 10mg of gelatin (Sigma, Steinheim G-8150), or 3mg of casein (Sigma, Steinheim, 6905) was added to the separating gel, and the mixture was incubated at 56°C for 15 minutes prior to pouring. All gels were prepared using the Biorad Miniprotean II vertical electrophoresis system (Biorad, California, 165 – 2940).

Samples (collected as described in 2.3.1.1 and 2.3.1.2) were diluted with the zymography buffer in a 4:1 ration. 6 µl of each sample dilution, as well as 6 µl of a cell-free control was loaded onto the polyacrylamide gels. In all gels, 5 µl of pre-stained SDS-PAGE standards based on the Laemmli method, was loaded to allow for the quantification of molecular weights. In addition, 6 µl of pre-prepared MMP-2/MMP-9 (kindly donated by Mrs Mona Bracher, CVRU, Cape Town, South Africa) was loaded onto gelatin-containing gels allow for confirmation. The gels were allowed to run at a constant current of 25mA.
Material | Preparation | Supplier/Product no.
--- | --- | ---
Zymography buffer | 200 mM Tris base | Sigma, Steinheim, T-6066
3% (w/v) SDS | Sigma, Steinheim, L3771-100G
0.1% (w/v) Bromophenol blue | Sigma, Steinheim, B-8026
40% (v/v) glycerol | Sigma, Steinheim, G-2025
pH 6.8

Tank buffer | 0.3% (w/v) Tris base | Sigma, Steinheim, T-6066
1.4% (w/v) glycine | BDH, Wadeville, RSA, B8101196N
0.1% SDS | Sigma, Steinheim, L3771-100G

2.3.3.2 Washing, Incubation and Detection

Gels were then washed twice in 2.5% Triton-X 100 (Sigma, Steinheim, T-8532) at 37°C for 15 minutes with agitation to remove traces of SDS. The gel was then transferred to a container with substrate buffer (see below), and was incubated for 18 hours at 37°C with agitation. After this, the gel was stained for 15 minutes in a detection solution (see below), and de-stained in de-staining solution (see below). The final gels were scanned on an Umax Astra 1220S Flat-Bed scanner at 100 dpi without compression and saved as TIFF files. The image inverted in Adobe Photoshop 7.0 so that the OD of the bands could be reported as positive values, and analysed using the NIH densometric analysis program (NIH image 1.60). Statistical analyses were performed as detailed in 2.3.2.3. All gels were dried using drying solution and stored for further reference.

Material | Preparation | Supplier/Product no.
--- | --- | ---
Substrate buffer | 50 mM Tris base | Sigma, Steinheim, T-6066
5 mM calcium chloride | ACE, Reuven, C0439NN00500
pH 8
Detection solution | 0.1% Coomassie Brilliant Blue R250 | Sigma, Steinheim, B-7920
42% methanol | Ilovo, RSA
16% glacial acetic acid | BDH, Wadeville RSA, BB100017P
Destaining solution | 15% methanol | Ilovo, RSA
7.5% glacial acetic acid | BDH, Wadeville, RSA BB100017P
Drying solution | 30% methanol | Ilovo, RSA
3% glycerol | BDH, Wadeville, RSA, BB101186M
2.3.4 Silver-Staining

Samples were separated on a 10% polyacrylamide gel, as described above (2.3.2.1). Following separation, the gels were analysed by silver staining, using the BioRad Silver Stain Plus™ kit (Biorad, Hercules CA, 161-0449). Briefly, gels were fixed for 20 minutes in fixative enhancer solution (see below) with gentle agitation at room temperature. The eifative enhancer solution was then decanted, and the gel rinsed in 400 ml of deionized distilled water for 10 minutes with gentle agitation. This rinse step was repeated three times. The gel was then stained for 20 minutes with staining solution (see below). When the desired staining was reached, the gel was placed in 5% acetic acid to stop the reaction. After 15 minutes in acetic acid (BDH, Wadeville RSA, BB100017P), the gels were rinsed in high purity water for 5 minutes. The gels were then scanned using scanned on an Umax Astra 1220S Flat-Bed scanner at 100 dpi without compression and saved as TIFF files. The images were analysed as described in 2.3.2.3.

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fixative enhancer</td>
<td>50% Reagent grade methanol</td>
<td>Ilovo, RSA</td>
</tr>
<tr>
<td>solution</td>
<td>10% Reagent grade acetic acid</td>
<td>BDH, Wadeville RSA, BB100017P</td>
</tr>
<tr>
<td></td>
<td>10% Fixative enhancer concentrate</td>
<td>Biorad, Hercules CA, 161-0461</td>
</tr>
<tr>
<td></td>
<td>30% Deionized distilled water</td>
<td></td>
</tr>
<tr>
<td>Staining solution</td>
<td>10% Silver complex solution</td>
<td>Biorad, Hercules CA, 161-0462</td>
</tr>
<tr>
<td></td>
<td>10% Reduction moderator solution</td>
<td>Biorad, Hercules CA, 161-0463</td>
</tr>
<tr>
<td></td>
<td>10% image development reagent</td>
<td>Biorad, Hercules CA, 161-0464</td>
</tr>
<tr>
<td></td>
<td>70% Deionized distilled water</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immediately before use add 100%</td>
<td>Biorad, Hercules CA, 161-0448</td>
</tr>
<tr>
<td></td>
<td>development accelerator solution</td>
<td></td>
</tr>
</tbody>
</table>

2.3.5 PDGFR-β ELISA

PDGFR-β expression in monocyte cell lysates was assayed using a PDGFR-β ELISA kit (R&D Systems, Minneapolis). Monocytes were isolated from whole blood and cultured for 15 hours in serum-free RPMI-1640 containing Ang-2 (500 ng/ml) and/or PDGF-BB (15 ng/ml). The cells were then lysed (as in 2.3.1.4) and the concentration of protein in the cell lysates tested as above. The ELISA was then performed on equal quantities of cell lysates according to manufacturers specifications. Human dermal fibroblast cell lysates were used as a positive control.
2.4 Analysis of Gene Expression in Human Monocytes

2.4.1 RNA Isolation and Reverse Transcription

RNA samples prepared from monocytes incubated for 6 hours at 37°C in serum-free RPMI-1640. The RNA isolated using RNEasy Micro-RNA isolation kit (Qiagen). The final RNA suspension (~ 12 μl) was divided into four equal aliquots and immediately stored at -80°C. Prior to use, the concentration of each RNA sample was assessed using RiboGreen (Molecular Probes, Leiden, R-11490) according to manufacturer's recommendations.

40 ng of each RNA sample was subjected to reverse transcription, using the Promega ImProm-II reverse transcription system (Promega, Madison, A3800) and a Hybaid PCR express PCR machine programmed according to protocol specifications. Briefly, the experimental RNA was incubated for 5 minutes at 70°C with 0.5 μg Oligo(dT) primer (Promega, Madison, C110A). Following this, it was chilled in ice-water for 5 minutes, and centrifuged to collect condensation.

The RNA-primer aliquot was then added to the reverse transcription reaction mix containing components of the ImProm-II reverse transcription system in a microfuge tube on ice. The reaction mix contained ImProm-II 5x reaction buffer, MgCl₂ (2M), dNTP mix, Recombinant RNasin (Promega, Madison, N211A) Ribonuclease inhibitor, and ImProm-II reverse transcriptase.

The mixture was then annealed for 5 minutes at 25°C, and extended for one hour at 42°C. Following this, the reverse transcriptase was inactivated by heating the mixture to 70°C for 15 minutes. All heating steps were carried out using an automated PCR machine (PCR express, Hybaid).

2.4.2 Semi-Quantitative Polymerase Chain Reaction

Primers for the genes of interest were ordered from the Central Analytical Facility (CAF) at the University of Stellenbosch, Stellenbosch, South Africa. The primers were designed by the researcher using the Primer-3 web-based primer design software (www.genome.wi.mit.edu/rgib-bin/primer/primer3; Dec 2004 – July 2006). The sequences used for each primer pair are detailed in the table below.
<table>
<thead>
<tr>
<th>Gene of Interest</th>
<th>Left Primer</th>
<th>Right Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-actin</td>
<td>5’TGGGCATCGTCACAAACTG3’</td>
<td>5’AGGCGTAGAGGGACAGGA3’</td>
</tr>
<tr>
<td>HPRT</td>
<td>5’CCCCCTGGCTCGTGATTAGS’</td>
<td>5’GCTCCCCTCCCTCTCATC3’</td>
</tr>
<tr>
<td>MMP2</td>
<td>5’ATGACAGCTGACACCTGAGS’</td>
<td>5’ATTGCTGCCAGGAAGGTGS’</td>
</tr>
<tr>
<td>MMP3</td>
<td>5’GCAGTTGTCACCGCTATCC3’</td>
<td>5’GAGGTGGATGGCTCCAGCTTCS3’</td>
</tr>
<tr>
<td>MMP7</td>
<td>5’GAGGTGCGATGTCGGCAAA3’</td>
<td>5’AAATGCAGGGGATCTCTTTS3’</td>
</tr>
<tr>
<td>MMP9</td>
<td>5’TGGACAGCGAAAGAAGTGS3’</td>
<td>5’GCCATTCAGGCCTGTCCTTAT3’</td>
</tr>
<tr>
<td>MMP12</td>
<td>5’ACACATTTTGCTCTCTGCT3’</td>
<td>5’CCCTCAGGCGAGGAACCTG3’</td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>5’TAAACCCAAAAACCCACCT3’</td>
<td>5’GCCTCATAAACCCACCAAT3’</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>5’GCACTTTATCCACCCAGG3’</td>
<td>5’GTACCTGGCTCCGCTTCCAGS3’</td>
</tr>
<tr>
<td>Tie-2</td>
<td>5’TGGAGGAGGACAGAATCAGS3’</td>
<td>5’CTGAGCATGGCAGGGTGT3’</td>
</tr>
<tr>
<td>uPA</td>
<td>5’TGGAGATCTGAGGTTTGG3’</td>
<td>5’TTTTCATGGGCGCCAG3’</td>
</tr>
<tr>
<td>uPAR</td>
<td>5’TCACACACAAAAATGCTGTGT3’</td>
<td>5’AGGCATTCTCCCTCTGGT3’</td>
</tr>
</tbody>
</table>

Table 9. Primers used during semi-quantitative PCR

PCR cocktails were set up according to manufacturers specifications as detailed in the table below. 2 μl of each RT-PCR reaction was used for each PCR reaction.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Supplier</th>
<th>Working conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left and right primer</td>
<td>CAF, University of Stellenbosch, RSA</td>
<td>1 mM</td>
</tr>
<tr>
<td>MgCl2 free buffer (M1901)</td>
<td>Promega, Madison</td>
<td>1x</td>
</tr>
<tr>
<td>MgCl2 (A3511)</td>
<td>Promega, Madison</td>
<td>2 mM</td>
</tr>
<tr>
<td>dNTP mix (1969064)</td>
<td>Roche, Mannheim</td>
<td>2 mM</td>
</tr>
<tr>
<td>Taq polymerase</td>
<td>Promega, Madison</td>
<td>1.25 U</td>
</tr>
</tbody>
</table>

Table 10. Composition of PCR cocktail

The PCR cocktails were placed in an automated PCR machine (PCR express, Hybaid), and the reaction carried out under the following conditions for 35 cycles:

- Denaturation: 94 °C for 30 seconds
- Annealing: 60 °C for 1 minute
- Extension: 72 °C for 2.5 minutes
All PCR products were analysed on a 1% agarose (Whitehead scientific, Johannesburg, 1800) gel containing 0.1% ethidium bromide. The gels were visualized using the BioRad Geldoc 1000 system.

2.5 Analysis of Potential Signaling Molecules

2.5.1 Immunocytochemistry (ICC)

2.5.1.1 ICC Analysis of Selected Cell Membrane Proteins

Monocytes (isolated as in 2.1.1) were suspended in serum-free RPMI-1640 and seeded onto 8-well Lab-Tek Permanox chamber slides at 70000 cells per well. Permanox plastic is a solvent resistant polymer suitable for tissue culture and fluorescent applications (manufacturer's information). Ang-2 (500 ng/ml) and PDGF-BB (15 ng/ml) were added to specific wells, and the cells incubated for 18 hours at 37°C.

The primary antibodies from the Integrin Classics Investigator Kit (Chemicon International, Temecula, ECM435) were diluted 1/1000 according to manufacturer's specifications in PBS containing 1% BSA, and the solution incubated with the cells for 1 hour at room temperature. "Secondary-only" control slides were incubated with PBS containing 1% BSA. The primary solutions were then aspirated off, and the cells washed three times with PBS. The secondary antibody, also in PBS with 1% BSA, was added to the cells at concentrations of 1/50 (Cy3-tagged donkey anti-mouse IgG, Jackson, Suffolk, 715-166-150), and allowed to incubate for a further hour at room temperature in the dark. The cells were then washed three times with PBS, also in the dark.

Coverslips were then mounted onto slides with a drop of VektaShield Mounting Medium with DAPI (Vector laboratories, Burlingame, H-1299), and the coverslip sealed with nail polish to prevent drying and movement under the microscope. Prior to analysis, the slides were stored in the dark at 4°C.
2.5.1.2 Microscopy Analysis

2.5.1.2.1 Fluorescent Microscopy

A Zeiss axiovert 200 fluorescent microscope and Zeiss Axiocam (Department of Anatomy and Cell Biology, UCT, RSA) were utilized to visualize the slides produced in 2.5.1.2. Cells were visualized using a mercury vapour fluorescence lamp with DAPI and Cy3 filters. The DAPI filter has an excitation boundary of 358 nm and an emission boundary of 461 nm. The Cy3 filter has an excitation boundary of 550 nm and an emission boundary of 570 nm. All images were taken with a 40x objective. Images were captured and analysed using the Zeiss Axiocam imaging software. Images were saved as TIFF files.

2.5.1.2.2 Confocal Microscopy

Confocal microscopy was utilized to determine the exact position of the fluorescent tag in relation to the cell. A Leica DM IRBE confocal microscope and associated imaging software were used to optically section cells stained with Cy3 (prepared as described in 2.5.1.2).

Using a 63x water immersion lens, optical stacks consisting of 1.5 μm steps were taken through the sample using the software provided with a scan speed of 400 Hz. The pinhole was set at 1 Airy Unit, while the photomultiplier tube 2 (PMT2) were set at 3.53 (offset), 822.6V (voltage). AOTF (568) was 80%. The SP mirror 2 (emission window) was set at 589 nm (left), and 673 nm (right).

2.5.2 Immunoprecipitation

2.5.2.1 Sample Preparation

Monocytes isolated from whole blood (2.1) were seeded into 12 well plates (Corning, NY) and incubated with serum-containing RPMI-1640 for 5 hours at 37°C with 5% CO2. The medium was then drawn off and replaced with serum-containing RPMI-1640 with 500 ng/ml Ang-2. The cells were incubated for a further 8 hours.

The cells were then washed thoroughly with PBS to remove unbound Ang-2. 200 μl of lysis buffer (see below) was then added, the cells scraped with a rubber policeman, and incubated for an hour at 4°C. Following the incubation, the lysate was removed to a 1.5 ml eppendorf
tube, and the wells re-scraped with another 200 µl of lysis buffer. This was also added to the lysate-containing eppendorf.

The protein concentration of the cell lysates was then quantified using the BCA™ Protein Assay Kit (Pierce, Rockford, 23227). Briefly, according to manufacturer's specifications, 25 µl of each sample or standard (Albumin 2mg/ml, provided by manufacturer) were pipetted into a microplate well. 200 µl of the working reagent (see below) was added to each well, and the plate mixed thoroughly. The plate was then covered and incubated for 30 minutes at 37°C, after which it was cooled to room temperature. The absorbance was measured at 540 nm.

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis Buffer</td>
<td>1% Nonidet P40</td>
<td>BDH, RSA, 56009</td>
</tr>
<tr>
<td></td>
<td>1x Protease inhibitor cocktail</td>
<td>Sigma, Steinheim, P8340</td>
</tr>
<tr>
<td>Working reagent</td>
<td>50 parts BCA reagent A</td>
<td>Pierce, Rockford, 23227</td>
</tr>
<tr>
<td></td>
<td>1 part BCA reagent B</td>
<td>Pierce, Rockford</td>
</tr>
</tbody>
</table>

### 2.5.2.2 Immunoprecipitation

Cell lysates generated in 2.5.2.1 were added to an eppendorf tube along with 500 µl of purified water, and 3 µg of purified monoclonal mouse antibody against human integrin β2 (Abcam, Cambridge, ab657) was added to the samples. In control samples mouse IgG was added instead of the antibody (Abcam, Cambridge, ab9404). The contents of the eppendorf were then gently mixed, and incubated for an hour on ice to allow immune complexes to form.

In order to precipitate the immune complexes, 20 µl of Protein-G sepharose (see below) was added to the eppendorf. The mixture was then incubated at 4°C for an hour with gentle agitation. The antibody-antigen-Protein G complexes were collected by centrifugation (13000 RPM for 15 minutes at 4°C), and resuspended in 2x sample application buffer (see 2.4.2.1). These samples were then resolved on a 10% polyacrylamide gel and probed for Ang-2 or uPAR (see 2.3).

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein G-Sepharose</td>
<td>20 µl of Protein G-Sepharose was washed three times with 500 µl lysis buffer, and suspended in 20 µl lysis buffer.</td>
<td>Sigma, Steinheim, P23296</td>
</tr>
</tbody>
</table>
2.5.3 **PDGFR-β ELISA**

PDGFR-β expression in monocytes cell lysates was examined using a PDGFR-β ELISA kit obtained from R&D Systems (R&D Systems, Minneapolis, DYC385-2). Cells isolated as described in 2.1.1 were suspended in serum-free RPMI-1640 and seeded into 12 well plates at a concentration of $0.5 \times 10^6$ cells per well. The cells were then incubated for 15 hours at 37°C with 5% CO₂ with Ang-2 (500 ng/ml) and/or PDGF-BB.

Following the incubation procedure, the cells were washed three times in PBS, and lysed as described in 2.5.2.1. After an hour incubation at 4°C, the lysates were collected and spun for 10 minutes at 13000 RPM. The supernatants were then transferred to another eppendorf tube, and stored at -80°C until analysis.

The ELISA was conducted according to manufacturer's specifications. Briefly, an ELISA plate was coated with the capture antibody provided. After overnight incubation at room temperature, the capture antibody was washed off (3 x PBS) and the plate blocked with 1% BSA for 1 hour at room temperature. The plate was then washed as before, and the samples and standard added. After a 2-hour incubation at room temperature, the plate was washed as before, and the detection antibody added for 2 hours. The plate was then washed as before, and strepavidin added at the recommended concentration. After a 20-minute incubation at room temperature in the dark, substrate solution was added to the wells. Following another 20-minute incubation, the colour development was stopped with 1M sulphuric acid. The colour intensity was measured on a microplate reader (Benchmark Microplate reader, BioRad, California) at 450 nm, with an extinction wavelength of 540 nm.

2.6 **Extension of Findings to Other Models**

2.6.1 **Matrigel Invasion Assay**

Growth Factor-Reduced (GFR) Matrigel Matrix (BD Biosciences, Bedford, BD356230) is purified and characterized to a greater extent than original Matrigel Matrix, and has reduced levels of growth factors (except TGF-β). Of the growth factors that remain, all are in ng - pg/ml concentrations.
GFR Matrigel is soluble at temperatures lower than 4°C, and gels rapidly at 22°C to 35°C. This necessitated that the product was kept on ice before use, and pre-cooled pipettes and plates were used during the preparation process.

The serum-free fibrin invasion assay (2.2.7) was repeated using GFR Matrigel instead of fibrin. 35 µl of GFR Matrigel was pipetted onto the Transwell membrane and allowed to gel at 37°C for half an hour. These coated wells were placed in compartments containing serum-free RPMI. Monocytes isolated as described in 2.1.1 were re-suspended in RPMI-1640 and at a concentration of 50000 cells/well into the coated Transwells. PDGF (20 ng/ml) and Ang-2 (500 ng/ml) were then added to the bottom compartment. The Transwells were incubated for 4 days, with the cell invasion assessed every day as determined by the number of cells appearing in the bottom compartment. After 4 days the lower compartments were fixed with 2.5% gluteraldehyde, and analysed as described in 2.1.9.

2.6.2 Analysis of the Effect of Ang-2 and PDGF-BB on Other Cell Types

HSVEC were obtained and maintained as described in 2.2.3. Human fibroblasts were obtained from a human abdominoplasty sample and maintained as described. Human aortic SMC primary cultures were prepared from the intimal-medial layer of an aorta from a heart transplant donor as previously described (Ross 1980). The cells were characterized by α-SMA actin immunocytochemistry. Primary cultures of human dermal fibroblasts were prepared from a 7.5cm x 7.5cm abdominoplasty sample obtained from the operating theatre. All cells were isolated by Mrs Mona Bracher (CVRU, UCT, Cape Town, South Africa) and used under passage 4.

The fibrin invasion assay described in 2.2.1 was repeated with the three different cell types. Fibrin-coated Transwells were seeded with each of the three cell types at 80% confluence in MCDB-131 medium containing 20% HPS. To the endothelial cell medium, 10 ng/ml epidermal growth factor (Peprotech, Rocky Hill, NJ, 100-15) and 5 ng/ml basic-fibroblast growth factor (Peprotech, Rocky Hill, NJ, 100-42) were also added. Ang-2 (500 ng/ml) was then added to the bottom compartment of the experimental wells.

The Transwells were incubated at 37°C with 5% CO2. The fibrinolytic potential of the cells was monitored by regularly assessing the number of cells appearing in the bottom compartment as described in 2.1.9. Cells in the bottom compartment of Transwells in triplicate were fixed and quantified at 3 and 4 days for each cell type.
Reagent | Preparation | Supplier / Product no.
--- | --- | ---
MCDB-131 medium | MCDB medium 131 | Sigma, Steinheim, M-8537
1.18 g/l NaHCO₃ | Sigma, Steinheim, S-5761
Filtered with 0.22 µm low protein binding filter | Milipore, Bedford, SCGVTO1RE
2 mM L-glutamate |  | 
pH to 7.4 |  |

### 2.6.3 In Vitro Analyses with Animal Monocytes

#### 2.6.3.1 Analysis Using Rat Peripheral Blood Monocytes

Blood was taken from male 3-month old Wistar rats (which were scheduled to be culled) through exsanguination by cardiac puncture. These operations were kindly performed by Mr Noel Margraaf of the Animal Unit (UCT, Cape Town, RSA). The blood from three rats was pooled, and 10% trisodium citrate added. The monocytes were isolated from the blood as described in 2.1.1.

#### 2.6.3.2 Analysis Using Baboon Peripheral Blood Monocytes

Baboon blood was generously provided by the Cardio-Thoracic surgery department, UCT, South Africa. These baboons had been obtained by the department for an ethically approved study of cardio-implants. The baboons were caught in the wild, quarantined for 4 weeks, and then housed at the Medical Research Council Animal Centre at Delft, Cape Town, South Africa. While at the Animal Centre, the baboons were dosed with Vitamin B, Ivomec and tuberculin tested. The baboons were otherwise medication-free at the time of blood drawing.

All baboons were fully anaestatised in preparation for the aforementioned cardio-implant procedure. Prior to the commencement of this procedure, 50 ml of venous blood was drawn from the forearm of the baboon into a sterile syringe containing 5 ml of tri-sodium citrate, and monocytes were separated from the whole blood using a Histopaque gradient, as detailed in 2.1.1. The standard serum-free fibrin invasion assay (as in 2.2.1) was repeated with the isolated monocytes.
2.7 *In Vivo Analyses*

2.7.1 Preparation of Heparin Discs

Porous polyurethane (PU) discs coated with deaminated heparin were kindly supplied by Dr Deon Bezuidenhout and Mrs Anel Oosthuizen (CVRU, UCT, South Africa). Briefly, the PU discs were subjected to surface modification by grafting acrylic acid/acrylamide copolymers to the surface. This precursor step was then followed by surface modification with ethylene diamine, which added surface amines to the disc. To these modified discs, the deaminated heparin was then added. The reaction was tested using Toluidine Blue which, when added to the disc, causes it to become dark purple. An unsuccessful reaction would remain clear.

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylic Acid</td>
<td>MW 72.06</td>
<td>Aldrich, 147230</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>MW 71.8</td>
<td>Aldrich, 148660</td>
</tr>
<tr>
<td>Ethylene diamine dihydrochloride</td>
<td>MW 133.02</td>
<td>Fluka, 03580</td>
</tr>
<tr>
<td>Deaminated Heparin</td>
<td></td>
<td>Celsus Laboratories, 57282325</td>
</tr>
</tbody>
</table>

2.7.2 Analysis of Ang-2 Binding to Heparin

Ang-2 was diluted in sterile PBS to a concentration of 1 μg in 50 μl. This solution was placed in a sterile well of a 96 well plate. The heparin disc was dropped into the solution, and the plate incubated at 4°C overnight.

The following day, the saturated disc was taken out of the well and placed on a sterile surface. 10 μl of the fluid inside the disc was drawn off using a pipette. This was stored at -20°C for further analysis. The disc was then washed three times in 1 ml PBS (2.2.1) for 20 minutes at 37°C to remove any unbound Ang-2. The disc was then incubated in 1 ml of PBS at 37°C. The PBS was replaced at 12, 24 and 48 hours, and these aliquots stored at -20°C. Following the incubations, another 10 μl was taken from the disc and this aliquot, as well as the disc, were also stored at -20°C.

The aliquots of PBS were then freeze-dried for 2 days on Virtus benchtop freeze-drier after which they were rehydrated in 10 μl of PBS. The samples were separated by SDS-PAGE, after which the gels were analysed by using the Silver Stain Plus silver staining kit (as
described in 2.3.4) (Biorad, California, 161-046). Each blot was run with a standard of recombinant Ang-2 at a known concentration.

2.7.3 Coating Heparin Discs With Fibrin

Heparin discs were prepared with Ang-2 (1 µg) and PDGF-BB (1 µg). The growth factors were added to 50 µl of PBS and incubated with the discs overnight at 4°C. Holes were drilled in a 4 mm Teflon sheet with a diameter of 7.5 mm. This sheet was sterilized with 100% ethanol, and placed on a sterile petri dish. Fibrinogen (10 mg/ml) and thrombin were added to the holes in the Teflon sheet, and a disc containing the growth factors was gently inserted 3 mm into the gel, allowing a fibrin coating of 1 mm on all surfaces of the gel.

2.7.4 Analysis of Fibrin Coating Using Scanning Electron Microscopy

The fibrin coated discs were treated and analysed as described in 2.1.7.

2.7.5 In Vivo Analysis of Tissue Ingrowth into Fibrin-Coated Heparin Discs

Heparin-coated discs (2.7.1), filled with Ang-2 and/or PDGF-BB in PBS (2.7.2) and encapsulated in fibrin (2.7.3), were implanted subcutaneously in 3 month-old male Wistar rats (UCT Animal Unit, UCT, South Africa) by Dr Stephan Dobner. This procedure was approved by the animal ethics committee of UCT, as determined by the Treaty of Helsinki. Prior to the operations the rats were anaesthetized using ketamine and rampin by Mr Noel Markgraaf (Animal Unit, UCT, South Africa). Six rats were used in these experiments. In each rat 2 incisions were made on each side of the spinal cord. Into each rat a PBS-, a PDGF-BB-, an Ang-2-, and an Ang-2/PDGF-BB-containing disc were implanted. The order of the discs was rotated between the rats. The incisions were sutured closed and the animals returned to their cages. After 4 days the rats were euthanised by carbon dioxide by Mr Noel Markgraaf, and the discs explanted. The discs were fixed overnight in 3% paraformaldehyde, and then stored at 4°C in 70% ethanol.

2.7.6 Immunohistochemical Analysis of Implanted Heparin Discs

Discs were embedded into wax using the HistocentreZ (Shandon, Thermo-Fisher Scientific). The sample in the wax block was then cut into 6 µm sections and incubated for 1 hour at 60°C, and overnight at 37°C. The sections were then dewaxed and taken to water. Slides
were then covered with 1% BSA for 30 minutes, and rinsed 3x in PBS. The ED1 antibody (mouse anti-rat ED-1, AbD-Serotec, Raleigh, MCA341R) was diluted 1:1000 in 1% BSA and added to the slide. After 2 hours incubation, the slide was then washed 3x in PBS, and the secondary antibody (1:50, Cy3-tagged donkey anti-mouse IgG, Jackson, Suffolk, 715-166-150) added. The slide was then incubated for another 2 hours in the dark. The section was then coverslipped using VektaShield Mounting Medium with DAPI (Vector laboratories, Burlingame, H-1299).

2.7.7 Development of an Ang-2-Carrying Adeno-Associated Virus

2.7.7.1 Production and Use of Competent DH5α Cells

2.7.7.1.1 Preparation of Competent Cells

24 hours prior to the procedure, LB plates (see below) were streaked with DH5α obtained from the glycerol stocks maintained at -80°C by Mrs. Ronnett Seldon (CVRU, UCT, South Africa). 5 colonies were picked from the LB plates and inoculated into 100 ml of SOB medium (see below). The cells were grown for 2 hours at 37°C with continual shaking at 280 RPM. The flask was then transferred to an incubator maintaining a temperature of 18°C. The cells were grown with continual shaking (280 RPM) for approximately 25 hours until the OD₆₀₀ reached 0.6.

The cells were placed on ice for 10 minutes, and then spun down at 2000 x g for 10 minutes. The pelleted cells were re-suspended in 30 ml of ice-cold TB buffer (see below), and DMSO (Merck, Darmstadt, 802912) added to a final concentration of 7%. The suspension was incubated on ice for a further 10 minutes, after which the cells were aliquoted into 200 μL aliquots and snap-frozen in liquid nitrogen. The aliquots of competent cells were stored at -80°C until use.
**Material** | **Preparation** | **Supplier/Product no.**
--- | --- | ---
LB plates | 1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.1% NaOH (1M), 1.5% agar/agarose | Pronadisa, 1612.00
 | | | Pronadisa, 1702.00
 | | | Sigma, Steinheim, S-7653
 | | | Sigma, Steinheim, S-5881
 | | | Whitehead, RSA, 1800
 | | | Autoclave. Cool to 56°C
 | | | Add ampicillin to 100 µg/ml
SOB | 2% tryptone, 0.5% yeast extract, 0.05% NaCl | Pronadisa, 1612.00
 | | | Pronadisa, 1702.00
 | | | Sigma, Steinheim, S-7653
 | | | Autoclave
 | | | 1% KCl (0.25M) | Saarchem, RSA, 504202
 | | | 0.5% MgSO₄ (2M) | Saarchem, RSA, 4123920
 | | | 0.001% NaOH (5M) | Sigma, Steinheim, S-5881
TB Buffer | 0.3g Pipes, 0.22g CaCl₂, 1.864g KCl, 1.1g MnCl₂, pH to 6.7 | Sigma, Steinheim, P-1861
 | | | | ACE, Reuven, 39NN00500
 | | | | Saarchem, RSA, 504202
 | | | | ACE, Reuven, 880287623
 | | | | 0.22 µm filtered using low protein Milipore, Bedford, SCGVT01RE binding bottle-top filter

### 2.7.7.1.2 Transformation of Competent Cells

Aliquots of competent cells were removed from the -80°C and slowly defrosted on ice. 10 µl of recombinant DNA plasmids (see below 2.8.6.3) was added to each 200 µl aliquot, after which the cells were incubated on ice for 30 minutes. Following this incubation, the cells were heat-shocked for 40 seconds at 42°C. The tubes were then returned to ice, and 800 µl of pre-warmed SOC (see below) was added to each aliquot. The suspension of cells and medium was then incubated at 37°C for a further 40 minutes, after which the cells were plated LB agar containing 100 µg/ml ampicillin. The plates were incubated overnight at 37°C.

**Material** | **Preparation** | **Supplier/Product no.**
--- | --- | ---
SOC | SOB with 2% 1M sterile glucose | 74
2.7.7.1.3 Miniprep Analysis of Competent Cell Transformation

For each miniprep analysis, 5 ml of sterile LB medium was inoculated with a single bacterial colony from the plates (generated in 2.7.7.1.2), and grown to saturation overnight at 37°C whilst shaking at 280 RPM. 1 ml of each saturated bacterial suspension was stored at -80 °C in 2 ml eppendorf tubes with 1 ml 99% glycerol (Sigma, G-2025).

2 ml of the saturated bacterial suspension was aliquoted into a 2 ml eppendorf tube. The cells were pelleted by spinning the tube at 13 000 RPM for 5 minutes at 4°C. The supernatant was discarded, and the pellet re-suspended in 100 µl of GTE (see below). This suspension was incubated at room temperature for 5 minutes.

Next, 200 µl of NaOH (200 mM)/SDS (1M) (Sigma, L-3771) was added, and the suspension placed on ice for 5 minutes. 150 µl of potassium acetate solution (see below) was then added, and the contents of the tube vortexed at maximum speed to mix, and placed on ice for 5 minutes. Tubes were then spun for 3 minutes at 13 000 RPM to pellet the cell debris and chromosomal DNA. The supernatant was transferred to a fresh tube, and mixed with 800 µL of 95% ethanol. This was incubated for 2 minutes at room temperature in order to precipitate the plasmid DNA. After centrifuging the mixture for 1 minute at 13 000 RPM, the supernatant was removed, and the pellet washed with 1 ml of 70% ethanol.

After air-drying the pellet, the DNA was re-suspended in 50 µl of TE buffer (see below), and further purified using the phenol extraction method described below.

<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris/EDTA (TE)</td>
<td>25 mM TrisCl pH 8.0</td>
<td>Sigma, Steinheim, T-8066</td>
</tr>
<tr>
<td></td>
<td>10 mM EDTA</td>
<td>Sigma, Steinheim, ED355</td>
</tr>
<tr>
<td></td>
<td>autoclaved</td>
<td></td>
</tr>
<tr>
<td>Potassium acetate (5 M)</td>
<td>29.5% glacial acetic acid</td>
<td>BDH, RSA, BB100017P</td>
</tr>
<tr>
<td></td>
<td>KOH pellets to pH 4.8</td>
<td>Merck, Darmstadt, AB005033.500</td>
</tr>
<tr>
<td>GTE</td>
<td>TE with 50 mM glucose</td>
<td></td>
</tr>
</tbody>
</table>
2.7.7.2 Creation of Recombinant Plasmids

2.7.7.2.1 Phenol Extraction and Ethanol Precipitation of DNA

An equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, Sigma, Steinheim, P-3803) was added to the DNA suspended in TE (generated in 2.8.6.1.3). This mixture was vortexed vigorously for 10 seconds, and microcentrifuged for 30 seconds. Due to the visible phase separation in the microcentrifuged sample, it was possible to remove the aqueous phase with a 200 μl pipette. This was transferred to a new tube, and 1/10 vol. of 3 M sodium acetate (Saarchem, RSA, S821010) pH 5.2 was added. Next, 2.5 vol of ice-cold 100% ethanol was added, the sample vortexed, and incubated at -80°C for 15 minutes.

Following the incubation, the sample was microcentrifuged for 5 minutes, and the supernatant discarded. Following the addition of 1 ml 70% ethanol, this centrifugation step was repeated. The ethanol was then removed, and the pellet allowed to air-dry, before being resuspended in 30μl TE buffer. The plasmid samples were stored at -20°C.

2.7.7.2.2 Analysis of Plasmid DNA Using Restriction Enzyme Digestions

All restriction enzyme digestions were conducted utilizing a generalized protocol making use of restriction enzymes, buffers, and bovine serum albumen (BSA) obtained from Promega biological research products. In each digestion reaction, 10 μl of miniprep solution was added to a solution containing 13.7 μl nanopurified water, 3 μl of the appropriate digestion buffer, 0.3 μl BSA. To this mixture 2 μl of restriction enzyme, and 1 μl RNAse were added. The digestion mixtures were incubated for 2.5 - 3 hours at 37°C, after which an aliquot of the digestion was analyzed on an agarose gel.

<table>
<thead>
<tr>
<th>Restriction enzyme</th>
<th>Catalogue number</th>
<th>Buffer used</th>
<th>Length of digestion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bam H1</td>
<td>R6021</td>
<td>C</td>
<td>2.5 hrs</td>
</tr>
<tr>
<td>Eco R1</td>
<td>R6011</td>
<td>H</td>
<td>2.6 hrs</td>
</tr>
<tr>
<td>Not 1</td>
<td>R6431</td>
<td>D</td>
<td>3 hrs</td>
</tr>
<tr>
<td>Pst 1</td>
<td>R6111</td>
<td>D</td>
<td>2.5 hrs</td>
</tr>
<tr>
<td>Xba 1</td>
<td>R6181</td>
<td>C</td>
<td>2.5 hrs</td>
</tr>
</tbody>
</table>

Table 11. Restriction enzymes used during cloning
2.7.7.3 Production of AAV-GFP and AAV-Ang-2

2.7.7.3.1 Amplification of Genes

The GFP and Ang-2 genes were amplified by PCR (as in 2.6.2) using the primers below (made CAF, University of Stellenbosch, Stellenbosch, RSA). The pEGFP-C1 plasmid was used as a template for the GFP amplification, while cDNA (2.6.1) from endothelial cells was used for Ang-2 amplification. The primers were:

GFP
Left: 5' GCT GAT CTG AGT CCG 3'
Right: 5' GTG AAC CGT ATC CGC 3'

Ang-2
Left: 5' GCT GCT GGT TTA TTA CTG AAG AA 3'
Right: 5' TCA GGT GGA CTG GGA TGT TTA G 3'

The mixture was subject to a 25 cycle PCR (PCR express, Hybaid) with each cycle having a 55°C annealing temperature (1 minute); 72°C extension temperature (3 minutes); and a 95°C denaturation temperature (1 minute).

A fraction of the PCR product was analysed using a 1% agarose gel. The remainder of the reaction was purified using a PCR clean kit (Qiagen), and stored at -20°C.

2.7.7.3.2 Cloning of PCR product

The pDrive cloning vector is part of the Qiagen PCR cloning kit that exploits the single A bases present on PCR products amplified using Taq polymerase. Ligation reactions were conducted according to manufacturers specifications using a 2:1 ratio of PCR product to pDrive plasmid. The ligation reaction was incubated at 4°C for 30 minutes, and subsequently used to transform DH5α competent cells (as in 2.7.7.1.2).

The transformed cells were plated onto LB agar plates containing 100 μg/ml ampicillin, and incubated overnight at 37°C.

2.7.7.3.3 Miniprep Analysis of Ligation Efficiency

4 colonies from each plate generated in 2.7.7.3.2 were chosen at random and the subjected to the alkaline lysis miniprep procedure (2.7.7.1.3). 5 μL of each miniprep suspension was subjected to an Eco R1 (Promega, R6011) restriction enzyme digestion for 2.5 hours at 37°C.
The digestions were analyzed on a 1% agarose gel, which was run at 60 V for 30 minutes. Based on the results of the gel, an aliquot of the plasmid isolated from miniprep three was sent to the CAF, University of Stellenbosch for sequencing using primers to the T7 and SP6 promoters, which flank the position of the insert in the pDrive plasmid.

2.7.7.3.4 Cloning from pDrive into pCMV-MCS

AAV preferentially packages inserts of a certain size range. As the GFP insert was only 500 bp, it was necessary to use a pCMV-MCS containing a 700 bp stuffer fragment of nonsense DNA (fragment of pCI-neo) obtained from Mrs. Ronnett Seldon. Making use of this stuffered plasmid would ensure that the final size of the DNA insert of the AAV would be in the region of 1.2 kB. For the Ang-2 insert (1.5 kB) it was not necessary to use the stuffered pCMV-MCS.

Both the pCMV-MCS and the insert-containing pDrive plasmids were digested with two restriction enzymes for 3 hours at 37°C. For the GFP insert, Pst-1 and Xba-1 were used, while for the Ang-2 insert, Bam H1 and Xba1 were used. The digestion products were then separated on a 1% agarose gel, and the relevant bands excised and cleaned using a Qiagen gel purification kit.

Aliquots of both purified fragments were ligated together in a 5:1 (insert:vector) ratio overnight at 4°C using T4 ligase (Promega). 10 μL of the ligation mixture was used to transform a 200 μL aliquot of DH5α competent cells (2.7.7.1.2). The cells were grown overnight at 37°C, after which 10 colonies were analysed by alkaline lysis miniprep (2.8.6.1.3) and Eco R1/Pst1 restriction enzyme digestion.

2.7.7.3.5 Creation of pAAV-MCS Containing Recombinant DNA

Aliquots of both pCMV-MCS constructed in 2.7.7.3.4. and pAAV-MCS were purified using phenol extraction, and digested with Not1 for 3 hours at 37°C. Due to the small size differences between the resultant fragments in each digestion, the products were separated and analyzed using a 0.6% agarose gel (130 V for 5 hours). The appropriate bands were excised from the gel, and purified using a Qiagen gel purification kit.
The purified product from the pCMV-MCS digestion was further digested with alkaline phosphatase (Promega, Madison, M1831) for 0.5 hours at 37°C to prevent self-ligation. The alkaline phosphatase was subsequently inactivated by incubating the sample at 57°C for 10 minutes, after which the entire sample was purified using a Qiagen PCR purification kit.

The fragments of pAAV-MCS and pCMV-MCS were then ligated in a 1:2 ratio overnight at 4°C using T4 ligase. 10 μl of the ligation mixture was then used to transform a 200 μ aliquot of DH5α competent cells (2.7.7.1.2). The amplified plasmid was extracted by alkaline lysis miniprep (2.7.7.1.3), and analysed by Pst1 restriction enzyme digestion. The digestion was analysed on a 0.6% agarose gel.

2.7.7.3.6 Production of AAV Viral Stocks

HEK 293 cells were seeded at 3 x 10^6 cells per 100 mm tissue culture plate in 10 ml of DMEM growth medium 48 hours prior to transfection. The cells were only utilized for transfections once they had reached 80% confluence. Following the AAV helper-free system designed by Stratagene, 10μg of the recombinant pAAV-MCS (generated in 2.7.7.3.5) pAAV-RC and pHelper were added to 1 ml of 0.3 M CaCl₂ (ACE, RSA, 39NN0500) and mixed gently.

The HEK 293 cells were then transfected using the calcium phosphate transfection method. 1mL of 2 x HBS (see below) was aliquoted into a 15 ml tube, to which 1 ml of CaCl₂ (0.3 M) containing 10 μg of pAAV-MCS was added in a dropwise fashion. The HBS/DNA/ CaCl₂ was immediately added to a 100 mm plate of 80% confluent HEK 293 cells.

The plate was then incubated for 6 hours at 37°C, after which the medium was removed and replaced with 10 ml of fresh DMEM growth medium. The transfected HEK 293 cells were then incubated at 37°C with 5% carbon dioxide for 72 hours. Following the incubation, the cells were scraped from the plate into a 15 ml tube, so that the cells and the growth medium formed a suspension. This cell suspension was subjected to four rounds of freeze/thaw by alternating the tubes between liquid nitrogen and 37°C water bath.

The cellular debris was collected by centrifugation at 10 000 x g for 10 minutes at room temperature. The resultant supernatant was then aliquoted and stored at -80°C.
antigens have shown that there is substantial heterogeneity of phenotype (Gordon 2005). Further variation has been also been noted in monocyte activity between donors, for example in VEGF response to hypoxia (Schultz 1999), and inter-individual heterogeneity has been suggested resulting from environmental, epigenetic or genetic causes (Osnes 1994; Schultz 1999). As all in vitro analyses utilized freshly isolated monocytes obtained from a number of donors, considerable effort was made to standardize the monocyte isolation procedure and characterize the cells obtained.

Monocyte/macrophage migration in fibrin gels has previously been shown to be dependent on both fibrin and thrombin concentrations and on the nature of fibrin crosslinking (Ciano 1986). Bearing this in mind, the fibrin clots cast under experimental conditions were investigated for casting reproducibility and regularity.

3.1.2 Characterization of Human Peripheral Blood Monocytes

The purity of the monocyte populations derived whole blood by Histopaque density centrifugation and fibronectin adherence (2.1.1) can be variable if poorly isolated. It is possible that isolated monocyte populations can contain variable numbers of platelets, neutrophils and other leukocytes. This margin of foreign cells can be, to a large degree, contained by stringency of the washes during the centrifugation steps, and the removal of any neutrophil or platelets from the monocytes adhered to the fibronectin through vigorous rinsing prior to lifting the monocytes with PBSE. The suitability and reproducibility of the Histopaque isolation technique was tested using CD14 flow cytometry as well as by morphological analysis.

3.1.2.1 CD14 Flow Cytometry

CD14 is a membrane-associated glycosylphosphatiylinositol-linked protein expressed by monocytes and macrophages. CD14 acts as a co-receptor with TLR4 and MD2 for the detection of bacterial lipopolysaccharide, and is associated with monocyte activation (Goyert 1988; LeVan 2001). It is routinely used as a marker for monocyte populations in flow cytometry (Hubl 1995). Therefore, selected cell isolations from whole blood taken from a number of donors were analysed for CD14 expression (2.1.2) - thereby assessing the purity of the isolated monocyte populations (monocytes were assumed to be activated due to incubation on fibronectin). As shown in figure 6 below, 99.97% of the gated cell population were positive for CD14 expression, suggesting a high level of purity in the isolated
<table>
<thead>
<tr>
<th>Material</th>
<th>Preparation</th>
<th>Supplier/Product no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>2xHBS</td>
<td>280 mM NaCl</td>
<td>Sigma, Steinheim, S-7653</td>
</tr>
<tr>
<td></td>
<td>1.5 mM Na₂HPO₄</td>
<td>Sigma, Steinheim, S-9638</td>
</tr>
<tr>
<td></td>
<td>50 mM HEPES</td>
<td>Sigma, Steinheim, H-3375</td>
</tr>
<tr>
<td></td>
<td>pH adjusted to 7.1 immediately prior to use</td>
<td></td>
</tr>
</tbody>
</table>

2.8 **Statistical Analyses**

For each group of experiments, one-way ANOVA was performed when more than two groups were compared. A two-tailed Student *t*-test was used to assess differences between two groups. *P* < 0.05 was considered significant. Data was presented on bar graphs as mean values with error bars representing SEM.
3 Results and Discussion

3.1 Development of an In Vitro Fibrin Invasion Assay

3.1.1 Introduction

Monocytes are one of the first cell types to be recruited to the wound area during injury (Dvorak 1995; Jackson 1997; Croll 2004; Moldovan 2005). Once traversing the endothelial barrier, monocytes penetrate the fibrin clot and differentiate into macrophages (Duffield 2003). The mechanisms by which monocytes migrate into fibrin is an area of considerable research (Loscalzo 1996; Moldovan 2000; Bar-Or 2003; Levi 2004), and many of the molecules stimulating this migration have yet to be defined.

In order to investigate monocyte fibrin invasion in vitro a novel assay was developed in which commercially available Transwell permeable supports were utilized to suspend a fibrin clot in serum-containing medium. Monocytes seeded onto of the clot migrated through the fibrin and the semi-permeable membrane, and accumulated in the lower compartment of the Tranwell, thereby allowing for accurate quantification of the level of fibrin invasion. This fibrin invasion assay differed from other such assays in that the adaption of the Transwells allowed for relative ease of invasive cell quantification. While other invasion assays depend on manual counting of sprouts, tunnels or cells, the design of this assay allowed the number of invasive cells to be quantified using image analysis software, thus substantially reducing the potential for error. Before this assay could be utilized to investigate the effect of Ang-2 and PDGF-BB on monocyte invasion, however, it was necessary to standardize certain parameters, including the monocyte isolation procedure and the casting of the fibrin clots.

Heterogeneity of the macrophage lineage has long been recognized and is, in part, a result of the specialization of tissue macrophages in particular microenvironments. Circulating monocytes, which give rise to mature macrophages, are also known to be a heterogeneous population, although the physiological relevance of this is not completely understood (Gordon 2005). Monocyte morphology has shown considerable variation in size, granularity and nuclear morphology (Gordon 2005). In addition, studies of the mononuclear phagocytes using monoclonal antibodies specific for various cell-surface receptors and differentiation
populations. The gated population represented over 80% of the total number of events, with most of the other events occurring in the region corresponding with cell debris (personal communication Glenda Davison, Dept Haematology, UCT). These data indicated that the isolation technique employed produced adequately pure monocyte populations.

![Flow cytometry dot plot](image)

**Figure 6.** Representative dot plot of flow cytometry analysis of gated CD14 expression on monocytes. X axis denotes fluorescence (PE-conjugated anti-human CD14). Y axis denotes cell size. 96.87% of gated population (80% of total isolation) was positive for CD14.

As previously mentioned, monocytes are known to be a heterogeneous population with regard to size and granularity, as well as the expression of cell-surface receptors (Gordon 2005). This is reflected in the wide spread of the population across both axes.

### 3.1.2.2 Morphological Analysis of Isolated Monocytes

Monocytes appear on stained blood films as small cells (12 – 15 µm) with an eccentrically-placed nucleus occupying at least 50% of the cell's area (Ross 2002). When the cells were allowed to develop for 7 days, their change into macrophage morphology was visible. When visualized on by phase contrast microscopy (3.1.3), monocytes appeared as small, rounded cells. A representative phase contrast picture is shown in **Figure 7.** After 7 days, the immature monocytes had differentiated into macrophages. Their phenotype was seen to change from small and regular, to large cells with abundant cytoplasm and filamentous projections.

![Phase contrast microscopy](image)

**Figure 7.** Morphology of isolated monocytes. As visualized using phase contrast microscopy with 10x objective (as shown by arrow).
3.1.3 Standardization of the Isolation Protocol

Personal communications (Assoc Prof H. M. Wu, Ohio State Uni., USA; Assoc Prof P. Tipping, Monash Uni., Australia), as well as reports in the literature (Ernofsson 1996; Schultz 1999) suggest that the endogenous activity of monocytes differ both between donors, as well as within subsets of individual donors (Jin 2004). In order to standardize the donation criteria, monocyte fibrin invasion was repeatedly assessed under differing donation conditions.

As mentioned above, monocyte invasion into fibrin clots was tested using the novel fibrin invasion assay described in 2.1.9 of the materials and methods. Briefly, a fibrin clot was cast on top of the microporous membrane (8 μm pores) of a Transwell chamber. Cells were seeded on top of this clot, and allowed to migrate through it, through the membrane and collect in the bottom compartment. Experiments involving Transwell permeable supports are almost always quantified by counting the number of cells present on the underside of the semi-permeable membrane (Siegbahn 1990; Ernofsson 1996), however recent studies have suggested that counting the cells in the bottom well is a viable alternative method of quantification (Ellingsen 2000).

3.1.3.1 Analysis of Assay Quantification

In many studies (mostly of cellular chemotaxis) involving Transwell permeable supports cell movement was quantified by counting the number of cells present on the underside of the polycarbonate membrane (such as (Siegbahn 1990)). After incubation the cells and the remaining on the upperside of the membrane are wiped off with PBS, and the remaining cells stained with haemotoxylin and counted manually using light microscopy (2.2.3).

Transwells with the largest pore size (8 μm) were selected for these experiments, in order to allow the monocytes (12 – 15 μm diameter) free movement from the membrane into the lower compartment. Previous studies of in vitro migration of mononuclear cells in modified Boyden chambers (Ellingsen 2000) suggested that increasing the pore size of the membrane from 5 μm to 8 μm resulted in an increase in the number of non-adherent cells. Therefore, the cells present in the bottom compartment were also investigated as an alternative form of quantification. These cells were quantified by automatically counting the cells in the bottom chamber using Leica Q-Win software package (2.1.9).

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Figure 8 below is a representation of the different quantification methods. The diagram represents the figures obtained from counting both the underside of the membrane (represented as average number of cells per field) and the bottom compartment (total number of cells) from 2 different donors (experiments done in triplicate). As in the donors represented in the graph, the results from most donors exhibited around a 16x increase in cell numbers between the membrane and the bottom compartment quantification techniques. The similar fold increase in cell numbers between the quantification techniques in most donors suggested that both methodologies were adequate for the quantification of these fibrin invasion assays.

![Figure 8. Comparison between quantification methods for fibrin invasion assay. A schematic representation of the comparison between quantification methods in two different donors (in triplicate). Columns 1 and 3 represent the average number of cells per field on the underside of the semi-permeable membrane. Columns 2 and 4 represent the total number of cells in the bottom compartments. In donor 1, the number of cells in the bottom compartment is 17x greater than in a field of the membrane (columns 1 and 2), while in](image_url)

When the two methods of quantification were compared, counting the cells on the membrane appeared as a less viable alternative for two reasons. Firstly, the amount of force needed to clear the remains of the fibrin clot from the upper side of the membrane in order to mount the membrane on a slide meant that it was possible for cells on the underside of the membrane to be dislodged during the clearing process. Secondly, no suitable image analysis software was available to enable the cells on the membrane to be counted automatically, as the small, spherical nature of the cells in comparison to the numerous pores present in the membrane made cell identification difficult. This necessitated the researcher selecting fields and manually counting the number of cells in each field. As counting the cells in the bottom
compartment necessitated no manipulation of the cells, and utilized automated counting software; this method of quantification was used for all further experiments.

In order to confirm that the structures being counted using the automated Q-win software were cellular, the initial fibrin invasion assay quantifications were accompanied by DAPI treatment of the cells in the bottom compartment. As shown in the representative images below (Figure 9), 91% of the structures visible in the bottom compartment under phase contrast (Figure 9A) were represented as signals in the DAPI channel (Figure 9B). This significant correlation between the two images led to the conclusion that the structures being counted by the Q-Win software were cellular in nature, and that the counting method employed provided a suitable reflection of cells present in the bottom compartment.

![Image A](image1.png)

**Figure 9.** DAPI analysis of structures detected by the Q-Win software. A. Representative phase contrast picture of structures in bottom compartment of Transwell after migration through fibrin clot. B. Corresponding fluorescence image of (A) illuminated on the DAPI channel, indicating that at least 90% of structures seen in (A) are cellular.

Additional attempts were made to quantify the invasive cells in the bottom compartment by using alternative methods of cell counting, such as a haemocytometer, a Coulter counter and flow cytometry. For all these methods, it was necessary to collect the cells present in the bottom compartment prior to quantification. The low number of cells in the bottom compartments, as well as the propensity of the monocytes to stick to the plastic of the bottom compartment resulted in highly variable cell counts. The use of Corning Ultra Low Attachment (Corning, NY) cell plates (Shen 2001) in conjunction with the Transwell inserts did not additionally facilitate the collection of cells.

### 3.1.3.2 Cell Lines and Cryopreservation

The monocyte isolation procedure described in 2.1.1 is a very time-consuming protocol taking over 7 hours. It was therefore hoped that experiments could be conducted using either
cryopreserved human monocytes, or a monocyte cell line in order to minimize time expenditure.

Many different cell types have been immortalized into cell lines, allowing researchers the option of using the hardier cell line alternative as an approximation for the primary cells in experiments. A survey of existing literature revealed that the use of the available monocyte-like cell lines in monocyte studies was rare. It was speculated that this was due to the fact that most, if not all, existing monocyte-like cell lines are not actual immortalized monocytes, but rather cells from other origins which displayed monocyte-like characteristics. CCL46 (a kind gift from Prof Lutz Thilo, UCT), a monocyte-like mouse cell line investigated for possible use in these experiments was of lymphoma origin. Preliminary comparative experiments of fibrin clot invasion between this cell line and monocytes isolated from whole blood (2.1.1) proved unsatisfactory, as the cell line failed to invade the fibrin clot (Figure 10A). This suggested that the behavioural patterns for the two cell types were very different, and that using the CCL46 cell line was not appropriate.

Investigations with cryopreserved human monocytes (cryopreservation described in 2.1.5) correlated with references in the literature of reduced cell activity (Osnes 1994), and displayed much lowered viability in culture. Therefore, based on these observations, in all further experiments monocytes were isolated from fresh whole peripheral venous blood directly before experimentation.
3.1.3.3 Isolation Criteria

The protocol for the isolation of monocytes from whole venous blood involves separation of these cells from other cell types using a combination of density centrifugation and preferential adherence of monocytes to fibronectin. Although fibronectin is known to be an activating factor for monocytes (Xie 1998), its use in the isolation processes was unavoidable. As all cells used in experimental and control treatments were isolated in the same way, it was believed that the impact of this activation on experimental results would be minimized. Alternative isolation processes, such as the Miltenyi Biotech (Gladbach, Germany) Cell Separation kits, were investigated as possible substitutes, however no viable alternative was found due to the prohibitive costs of these and other commercially available kits.

It was found that the number of monocytes isolated from the same volume of whole blood could differ by several million cells from one donation to the next. 5 million cells were therefore taken as the average yield for an isolation of monocytes from 50 ml of whole blood (personal communication, Mrs Mona Bracher, UCT, South Africa), and isolations with yields of below 2.5 million cells, or above 7.5 million were discarded.

Figure 10. Monocyte-like cell lines and fibrin invasion. A. While freshly isolated monocytes (column 1) showed considerable fibrin invasion under serum-containing experimental conditions, CCL46, the monocyte-like cell line, showed no observable fibrin invasion over the same time (column 2). n = 2.
3.1.3.4 Demographic Criteria

It has been suggested that the endogenous activity of monocyte differs both between donors, as well as within subsets of individual donors (Ernofsson 1996; Schultz 1999; Jin 2004). This information suggested that it would be beneficial to select all donors from a similar demographic. Therefore, all donors used for experiments were males (all white except 1 Indian and 1 black) of between 22 and 29 years of age. For all donors, blood was taken at least three times in order to allow for internal comparison of cell functioning between experiments (as determined by fibrin invasion assays 2.1.9). Although all the donors used in the experiments presented in this thesis were male, many of the experiments were also repeated using female donors, to determine whether the results were sex-specific. Figure 11 indicates that average fibrin invasion was not a sex-specific characteristic, and that monocytes isolated from female donors invaded fibrin in a similar manner to those isolated from males.

![Figure 11: Comparison between monocytes isolated from male or female donors](image)

Figure 11. Comparison between monocytes isolated from male or female donors. The graph represents the average number of cells present in the bottom compartment after the cells were subjected to the fibrin invasion assay. The amount of fibrin invasion between monocytes isolated from females (column 1) and males (column 2) displayed similar invasive profiles.

\[ p = 0.62, \ n = 2 \] Experiments in triplicate.

Monocyte activity has previously been reported to be influenced by a number of exogenous factors, including stress and sleep deprivation (Savard 2003), health (Schultz 1999) and diet. These parameters were explained to each donor, and donors were routinely asked to report any such lifestyle changes. In addition, in an attempt to limit the possible influence of stress
on the study, any blood donation during which the procedure caused reported discomfort was not used in experiments.

Aspirin is known to have analgesic and anti-inflammatory properties, and is also an inhibitor of platelet aggregation. It inhibits fatty acid cyclo-oxygenase by acetylation of the active site of the enzyme, and the pharmacological effects of aspirin are due to the inhibition of the formation of cyclo-oxygenase producers including prostaglandins, thromboxanes and prostacyclin, thus affecting monocyte activity (Gordon). Potential donors who had taken aspirin (in the form of tablet, cough syrup, supplement or otherwise) were taken off any study for at least three weeks in order to allow the aspirin to clear from their system.

Lastly, a personal communication (Assoc Prof P. Tipping, Monash University, Australia), as well as references in the literature suggested that certain elements of the blood with cyclically varying concentrations, such as glucocorticoid and prostaglandin levels (Shapiro 1993; Lee 1995; Morand 1999), as well as PAI-1 (Dobrovolsky 2002), could affect the functioning of monocytes. Studies suggest that glucocorticoids influence the expression of many different regulatory molecules, including the PDGFs and their receptors, uPA, stromelysin-2 and MMP12 (Beer 2000; Esmon 2001). Indeed, it was noted that donations from the same donor at different times of day could cause moderately different results in identical experiments. All blood was therefore taken within ten minutes of 11:00 am.

3.1.4 Preparation and Analysis of Fibrinogen and Fibrin Gels

3.1.4.1 Analysis of Background Fibrinolysis

In vivo, fibrinogen, a major component of plasma, polymerises in the presence of platelet degranulation, leading to formation of a fibrin clot, which is believed to serve as an extracellular matrix enabling migration of cells such as endothelial cells and monocytes. In vitro, a fibrin clot can be formed by adding thrombin to fibrinogen (in the presence of calcium), both of which are commercially available. The fibrinogen was purified and prepared as described in section 2.1.6 and the thrombin according to manufacturer’s specifications.

Unfortunately, as a plasma protein, fibrinogen isolations (even those done commercially) are often contaminated with other plasma proteins, the most important of which is the plasminogen, the zymogen of the fibrin(ogen)-degradative enzyme plasmin. While
plasminogen-depleted fibrinogen was purchased for the purpose of these experiments, it was necessary to test all batches of fibrinogen used for possible plasminogen activity by Mrs Mona Bracher (CVRU, UCT, Cape Town, South Africa).

In addition, the addition of human pooled serum to culture medium increases the amount of plasminogen present in the experimental milieu (Jockenhoevel, Zund et al. 2001), making the standardization of baseline fibrinolysis in both cell-free and cell-containing experimental conditions vital.

Plasmin is known to degrade fibrin(ogen) into a number of different fibrin degradation products (FDPs) (Bini 1999). One of these major plasmin-dependent fibrinolysis by-products is a D-dimer fragment composed of two 90kDa monomers (Bini 1996; Bini 1999). Due to its prevalence in plasmin-dependent fibrinolysis, the relative quantity of D-dimer FDPs in a supernatant is commonly taken as a measure of plasmin activity. Commercially available D-dimer ELISAs have been designed for both clinical and laboratory use.

As described in section 2.1.8, a D-dimer ELISA was performed on selected samples in order to determine the contributions that the fibrinogen, serum and cells made to the baseline fibrinolysis. Fibrin clots were incubated in 96 well plates in either serum-containing (SCM) or serum-free medium (SFM). Freshly isolated human peripheral blood monocytes were seeded onto some of the clots. After overnight incubation at 37°C the conditioned medium was drawn off and the ELISA conducted on the samples. Figure 12 below presents a summary of the results.
Despite reports of spontaneous degradation of fibrin in culture medium (Jockenhoevel 2001), cell-free (CF) preparations of either serum containing (Figure A, CF SCM, column 1) or serum-free (CF SFM, column 2) medium displayed very low levels of fibrinolysis, with the D-dimer concentrations being 543 ng/ml and 527 ng/ml respectively.

As expected, the presence of macrophages increased the levels of fibrinolysis considerably, presumably through the activation of plasminogen by monocyte-produced plasminogen activators (Gyöko 1994). This increase in fibrinolytic potential was reflected in the D-dimer concentrations of the cell-containing (CC) samples with either serum-containing (16198 ng/ml, CC SCM, Figure 12A, column 3) or serum-free (3036 ng/ml, CC SFM, Figure 12A, column 4) medium. It is of interest to note that the contribution of serum to the monocyte fibrinolytic potential was significant, raising the D-dimer concentration by 5 times in comparison with that obtained in serum-free conditions. This suggested that in the fibrin invasion assay, the amount of cells passing through the fibrin would be higher under serum-containing than serum-free conditions.

Figure 12B displays representative photographs of the wells after the conditioned medium tested used in 12A had been removed. The remaining fibrin was stained with haematoxylin.
The arrows represent zones of lysis where the fibrin had been degraded. The zones of lysis are clearly visible in the cell-containing samples (Figure 12B, CC-SCM and CC-SFM), corresponding with the increase in D-dimers detected in the conditioned medium.

The D-dimer ELISA demonstrated that a baseline level of fibrinolytic activity was present when monocytes were seeded onto fibrin. It was therefore decided that any increase in fibrinolysis would be calculated as a percentage increase over the specific controls (discussed later in the text) set up for each individual experiment.

### 3.1.4.2 Analysis of Casting Reproducibility

Macrophage migration in fibrin gels is dependent on both fibrin and thrombin concentrations and on the nature of fibrin crosslinking (Ciano 1986). Fibrin concentrations of more than 3 mg/ml were seen to inhibit macrophage migration, while thrombin concentrations of 1 U/ml favoured maximal macrophage migration under the study’s experimental conditions.

![Figure 13A. Scanning electron microscope image of fibrin gel. 100x image](image_url)
The reproducibility of the fibrin gel casting, as well as the orientation of the fibrin strands under experimental conditions was assessed using scanning electron microscopy (2.1.7) (with the kind help of Ms Nazlia Samadion (CVRU, UCT, South Africa)). As can be seen from the representative images above (Figure 13, A: 1000x, B: 2000x), under experimental conditions fibrinogen (2.1.6) polymerized into a regularly oriented layer. This analysis was repeated with every batch of fibrinogen and thrombin used.

3.1.5 Summary

In order to investigate monocyte fibrin invasion in vitro, a novel Transwell assay was developed in which a fibrin clot was suspended in medium by a semi-permeable membrane. Cells seeded on top of the clot were able to pass through the clot and the membrane, allowing for the level of cell invasion to be quantified by the number of cells collecting in the bottom compartment after incubation.

To ensure assay reproducibility, considerable effort was made to standardize the experimental setup. As in vitro analyses are dependent on well-characterized cells from reliable sources, the monocyte isolation methodology was tested with regards to factors such as isolation purity, cryopreservation, and the influence of certain external factors such as sex, lifestyle and time of blood donation. Of interest, it was noted that in keeping with the literature (Lee 1995; Savard 2003), the activation (as determined by fibrin invasion) of monocytes varied among experiments involving monocytes donated at different times by the same individual (data not shown). It has been suggested that as circulating cells, monocytes
are susceptible to changes within the blood, such as fluctuating glucocorticoid levels (Morand 1999). Furthermore, monocytes isolated from different donors showed variability with regards to fibrin invasion potency (as seen in 3.1.3.1).

The fibrinogen used to cast the fibrin clots was tested for background fibrinolytic activity in the presence and absence of human pooled serum. The fibrin gel orientations were also scrutinized using electron microscopy, in order to ensure reproducibility. Using the parameters defined in this section, it was determined that a high degree of reproducibility could be obtained using the novel fibrin invasion assay. This reproducibility allowed for the assay to be used in further investigations involving Ang-2 and PDGF-BB.

3.2 Analysis of Monocyte Fibrin Invasion in Response to Ang-2 and PDGF-BB

3.2.1 Introduction

Ang-2 and PDGF-BB are cytokines known to be present during the early stages of tissue injury and wound healing. Ang-2 is released from the Weibel-Palade bodies of activated endothelial cells (Fiedler 2004), while PDGF-BB originates from, amongst other things, degranulating platelets (Hosgood 1993). The early release of both these cytokines into the wound environment suggests that they would be present concurrently with the fibrin clot and the inflammatory response.

In this series of experiments monocyte fibrinolytic response to Ang-2 and PDGF-BB was assessed in vitro using the novel fibrin clot invasion assay discussed in section 3.1. Monocyte invasion into these clots in vitro was first investigated under serum-containing conditions similar to those occurring during clotting and haemostasis. These experiments were then repeated in serum-free conditions in order to further analyse the nature of the effect.

When investigating an interaction in vitro, it is vital for physiological conditions to be maintained as far as possible. References in the literature of concentrations of Ang-2 used in vitro were noted to vary considerably according: such as 100 ng/ml (bovine microvascular endothelial cells (Mandriota 1998)), 500 ng/ml (human dermal microvascular endothelial cells (Hawighorst 2002)), 1 μg/ml (human umbilical vein endothelial cells (Witzenbichler 1998)), 2 μg/ml (murine microvascular endothelial cells (Mochizuki 2002)). Therefore, prior to in vitro experimentation, a working concentration of Ang-2 was determined by dilution
Monocyte migration into fibrin clots remains a subject of debate, and different studies have alternately shown their dependence on both plasmin (Loscalzo 1996; Levi 2004; Murdoch 2004) as well as MMPs (Moldovan 2000; Bar-Or 2003) during fibrinolysis. It was therefore imperative that the mechanism(s) of fibrinolysis be identified under these experimental conditions. Indeed, the plasminogen/plasmin- and MMP-systems have also previously been shown to be interdependent (van Hinsbergh 2006) and concurrently operational (Collen 2003), making it possible that any fibrinolytic activity seen was a combination of the two systems (Hiraoka 1998; May 2005). Initial experiments into the nature of the invasion observed in these experiments was therefore conducted.

3.2.2 Analysis of Monocyte Fibrin Invasion in Response to Ang-2 in a Serum-Containing Milieu

As very little research has previously been conducted on the interactions between Ang-2 and monocytes, the effect of Ang-2 was first investigated in a serum-containing environment, which resembled aspects of the wound-healing environment. The human pooled serum used in these experiments was prepared from commercially acquired human plasma, which had been clotted for 24 hours, so as to allow for maximum platelet degranulation and release of stored factors, such as PDGF-BB (Hosgood 1993; Hoyle 1999). Using this serum in the experiments allowed for the effect of Ang-2 to be assessed in an in vitro environment resembling aspects of the wound milieu, without the need to alter too many other parameters. As Ang-2 is known to be released by from the Weibel Palade bodies upon stimulus by triggers such as hypoxia (Mandriota 1998; Yuan 2000; Fiedler 2004), very little of this growth factor was present in human pooled serum. Recombinant Ang-2 was therefore added to the experimental wells.

3.2.2.1 Ang-2 Increases Monocyte Fibrin Invasion in a Serum-Containing Milieu

In order to determine the optimal Ang-2 concentration from the suggested concentration range mentioned above, a dose response analysis was conducted using Ang-2 concentrations from 100 ng/ml (Mandriota 1998; Witzenbichler 1998) to 1 µg/ml (Witzenbichler 1998). As indicated in Figure 14A, only concentrations of Ang-2 greater than 250 ng/ml caused a
significant increase in the number of monocytes present in the bottom chamber of the invasion assay. Based on the dosage curve analysis, 500 ng/ml was selected as the Ang-2 concentration for further experiments.

Figure 14. Relative increase in monocyte fibrinolysis due to the addition of Ang-2 to the invasion assay.
A. A dose response curve for Ang-2 showing the increase in the number of cells in the bottom compartment when cells are exposed to increasing amounts of Ang-2. *p < 0.05 (compared to concentrations ≤ 250 ng/ml). Experiment in triplicate (1 donor).
B. Summary of the increase in the number of cells in the bottom compartment when Ang-2 (500 ng/ml) is added to the bottom compartment. *p < 0.001. 9 experiments in triplicate (5 donors).
Once a suitable *in vitro* Ang-2 concentration had been determined, the invasion assay described in 2.1.9 was repeated on 5 donors in 9 independent experiments in triplicate. In these experiments it was observed that the presence of Ang-2 in the bottom chamber resulted in a 60% increase in the number of monocytes migrating through the fibrin clot, relative to media containing serum alone (Figure 14B, p < 0.001). In all experiments, clear evidence of fibrinolysis could be visually discerned in both the experimental and control wells, with the zones of lysis (such as described in 3.1.4.1) present in the fibrin in the Ang-2-containing wells being larger, therefore suggesting heightened fibrinolysis activity.

These experiments suggest that Ang-2 operates in a serum environment to promote monocyte invasion into fibrin. It was observed that after incubation, zones of lysis similar to those observed in 3.1.4.1 could be seen in the fibrin clot, suggesting that the fibrin invasion was occurring through some fibrinolytic mechanism, upregulated by the presence of Ang-2.

### 3.2.2.2 Analysis of the Chemotactic Potential of Ang-2

A number of different growth factors have lately been shown to exert a chemotactic effect on monocytes, such as MCP-1, TGF-β (Gyetko 1994; Sage 2002). No previous investigations have been conducted on the possible effect of Ang-2 on monocyte chemotaxis, although Ang-2 was not seen to exert a chemotactic effect on endothelial cells (Witzenbichler 1998). The manner of quantification of the fibrin invasion assay described above (3.2.2.1) made it imperative to ascertain whether Ang-2 had a chemotactic effect on monocytes. If monocytes were stimulated by an Ang-2 chemotactic gradient, counting the cells in the bottom compartment would not be an accurate representation of Ang-2 dependent monocyte fibrin invasion.

The Ang-2 fibrin invasion assay described above (3.2.2.1) was therefore repeated, however Ang-2 was placed in the top compartment instead of the bottom one, thereby reversing any Ang-2 concentration gradient. It was reasoned that the repetition of the results reported above in figure 14B would suggest that the Ang-2-stimulated fibrin invasion observed was independent of an Ang-2 concentration gradient. When Ang-2 was added to the upper compartment of the Transwell inserts, 4 experiments in triplicate with 3 donors showed that the cells in the bottom compartment increased by an average of 49% (Figure 15, p < 0.01). The resultant increase in monocyte movement in response to Ang-2 present in the upper
compartment differed non-significantly from that elicited by Ang-2 in the bottom compartment.

**Figure 15. Ang-2 does not influence monocyte chemotaxis under experimental conditions.** Schematic summary of the average increase in cells in the bottom compartment when Ang-2 (500 ng/ml) was added to the top compartment. *p < 0.01. 4 experiments in triplicate (3 donors).

In order to confirm the results observed in figure 15, another chemotaxis assay was conducted using an experimental model (see 2.2.3) previously used in a number of monocyte chemotaxis studies (Siegbahn 1990; Ellingsen 2000). Also using Transwell inserts, the monocytes were suspended on the semi-permeable membrane in a chemotactic gradient along which they are free to move without any impediment. The semi-permeable membrane facilitates the movement of cells in response to the concentration gradient, allowing for the potency of the gradient to be quantified by counting the number of cells present on the underside of the membrane after incubation.

In these experiments, monocytes were seeded onto the semi-permeable polycarbonate membrane (5 μm pores), and incubated for 4 hours under serum-free conditions in an Ang-2 (500 ng/ml) concentration gradient. Due to the smaller pore size, the chemotaxis experiments were quantified by counting the cells on the underside of the membrane (Ellingsen 2000). As described in 2.2.3, 5 representative fields from each of the triplicate experimental and control membranes were chosen at random under 10x magnification, yielding an average number of cells per field as displayed on the graphs below.
Figure 16 displays the results of the chemotaxis investigation. Based on the results obtained from the two independent chemotaxis experiments, it was conjectured that under the experimental conditions, Ang-2 exerted no chemotactic force on human peripheral blood monocytes in the presence or absence of serum. It was hypothesized that the movement of cells observed was due to random cell migration (chemokinesis), as the migration numbers were equivalent in the experimental and control membranes. Furthermore, these observations confirmed that the method of quantification of the fibrin invasion assay was appropriate, and that cells appearing in the bottom compartment after incubation were not attracted by a chemotactic gradient.

3.2.2.3 Analysis of the Proliferative Potential of Ang-2

As the monocyte fibrin invasion in response to Ang-2 was quantified by counting the number of cells present in the bottom compartment after incubation with the growth factor, it was also necessary to confirm that the positive increase observed in 3.2.2.1 was due to increased invasion, and not due to Ang-2-stimulated cell proliferation. Therefore, despite previous observations that circulating peripheral blood monocytes are not highly proliferative (Duffield 2003), as well as Ang-2 not being proliferative for endothelial cells (Witzenbichler 1998), the proliferative potential of Ang-2 on monocytes was tested using a cell proliferation ELISA (2.2.4).
The BrdU colorimetric assay allows cell proliferation to be measured using an immunoassay, which quantifies the incorporation of BrdU into the DNA of proliferating cells. As seen in figure 17 (p > 0.05 between columns 1 to 4), after 18 hours of incubation neither the untreated monocytes nor those treated with Ang-2 under serum-containing conditions exhibited proliferation significantly above the blank or background control. These data suggest that Ang-2 does not affect monocyte proliferation. The positive control (Figure 17, column 5) of endothelial cells with bFGF showed that the assay was functional.

This result confirms that the monocytes used in these experiments were not dividing, either with or without Ang-2. These results, together with those reported in 3.2.2.2 suggested that any increase in number of cells in the bottom compartment could be taken as a true reflection of monocyte fibrin invasion.

3.2.2.4 Ang-2-Induced Monocyte Migration Through Fibrin is Dependent on Both MMPs and Serine Proteases

Monocyte invasion into fibrin has previously been reported to occur through a number of different mechanisms, including plasmin-dependent (Blasi 1997; Levi 2004; Murdoch 2004), MMP-dependent (Moldovan 2000; Matias-Roman 2005), and non-fibrinolytic (Ciano 1986;
Lanir 1988) mechanisms. Broad-spectrum commercially available inhibitors were therefore utilized in order to facilitate an initial assessment of the mechanisms of the monocyte invasion observed in 3.2.2.1. In these experiments the Transwell invasion assay utilized in section 3.2.2.1 was modified to include the inhibitors in both the medium as well as the fibrin clot.

The following inhibitors were chosen for their use in identifying the mechanism of monocyte invasion under these experimental conditions: aprotinin, GM6001, and cycloheximide. Aprotinin is a serine protease inhibitor, and affects the functioning of both tPA and uPA as well as plasmin, while GM6001 is a peptide-based zinc-chelating hydroxyamate that acts as a broad-spectrum MMP inhibitor (Lund 1999). Cycloheximide is a protein-synthesis inhibitor, which binds to the 80S subunit of eukaryotic ribosomes and prevents the initiation of translation (Prager 2004). All inhibitors were used at concentrations recommended by the manufacturers.

Prior to the experiment, the possible cytotoxicity of the various inhibitors was assessed using the LIVE/DEAD® Viability/Cytotoxicity kit (Molecular Probes, Eugene, L-3224). Monocytes isolated as described in 2.1.1 were seeded into a 96 well plate, and exposed to the inhibitors for a 24-hour period. Following this incubation, the viability of the cells was tested as detailed in 2.2.5. At the concentrations used in these experiments, cell viability in the presence of each inhibitor was above 85 % (Figure 18).

**Figure 18.** Monocyte viability does not significantly decrease in the presence of selected inhibitors. Monocyte viability under experimental conditions (99%, column 1) does not significantly differ from viability under treatment with 6 mg/ml GM6001 (86%, column 2), 50 ng/ml aprotinin (88%, column 3) and 15 mg/ml cycloheximide (85%, column 4).
The addition of cycloheximide to the fibrin invasion assays knocked out all monocyte fibrin invasion in the presence of Ang-2 (Figure 19, column 4, p<0.05). These data indicated that Ang-2-stimulated monocyte invasion into fibrin is dependent on protein synthesis.

Interestingly, both proteolytic inhibitors significantly reduced the monocyte cell invasion in response to Ang-2. GM6001 repressed Ang-2-dependent cell invasion by 4 fold and aprotinin by 24-fold (Figure 19, column 3 and 4 respectively, p<0.05) demonstrating the involvement of both types of proteases. As demonstrated in the graph above, both the inhibition of the MMP and the plasmin(ogen) systems lowered fibrin invasion below that of the untreated cells. As results suggested, not only that monocytes utilize both fibrinolytic systems for the fibrin invasion seen in the control wells, but also that the heightened fibrin invasion seen in response to Ang-2 was due to the upregulation of existing fibrinolytic mechanisms.

Plasmin activation of various MMPs is well established (van Hinsbergh 2006), while MMP activation of the plasmin(ogen) system is beginning to be understood (Ugwu 1998; Collen 2003). Some research has shown a functional overlap between MMPs and the plasminogen/plasmin systems, and a requirement for both plasminogen deficiency and MMP inhibition for complete inhibition of the healing process in wounds (Lund 1999). Based on the observations above, however, it was not possible to determine whether in the presence of
Ang-2 and serum-derived factors, the upregulation of elements of both the plasminogen/plasmin and the MMP fibrinolytic systems involved degree of interaction between the two systems. Further investigations would be necessary in order to clarify this possibility.

3.2.3 Analysis of the Effect of Ang-2 on Monocyte Fibrin Invasion Under Serum-Free Conditions

The previous experiments established that Ang-2 affects monocyte fibrinolysis under conditions similar to those during haemostasis. Interestingly, when the invasion assays were repeated under serum-free conditions, Ang-2 did not have an effect on monocyte fibrinolysis. This observation suggested that Ang-2 was indeed acting in a context-dependent manner. The ability of Ang-2 to interact with other factors, such as VEGF has previously been reported, and it was likely that Ang-2 was interacting with an element of the human pooled serum (Mandriota 1998). PDGF-BB, as a product of platelet degranulation, seemed a likely candidate for investigation, as the human pooled serum utilized in these experiments was prepared from plasma which allowed to clot for 24 hours, so allowing for maximum platelet degranulation and release of such stored factors (Hosgood 1993; Hoyle 1999).

3.2.3.1 Analysis of Components of Human Pooled Serum

The human pooled serum used in these experiments was prepared from commercially acquired human plasma. Previous work done by Dr Mike Beck (Medtronic Inc, Minneapolis, USA, personal communication) analysed the concentrations of various growth factors in human serum. In this work, keratinocyte growth factor (KGF), bFGF, VEGF (74.1 pg/ml) and HBEGF (heparin-binding EGF-like growth factor) all were found to be at concentrations of less than 100 pg/ml. PDGF-BB, however, was seen to be at a concentration of over 3401 pg/ml.

As these data were obtained from single serum samples, the ELISA analyses were repeated on the human pooled serum used in all experiments reported in this thesis. PDGF-BB and VEGF were chosen as representative high- and low-concentration growth factors. When the ELISAs were repeated (2.2.6), the concentration of PDGF-BB in RPMI-1640 with 20% human pooled serum was found to be 851.11 pg/ml, while the concentration of VEGF was almost 100 fold lower, at 10 pg/ml. These data suggested that in the human pooled serum
utilized in these experiments, PDGF-BB was present at a concentration of 4255 pg/ml, while VEGF was present at 50 pg/ml. While the concentration of growth factors in the serum-containing medium samples tested differed slightly from those of the serum samples tested by Mike Beck, it is likely that this was due to variations between donors.

3.2.3.2 Ang-2-Mediated Fibrin Invasion Requires PDGF-BB Under Serum-free Conditions

Under serum-free conditions PDGF-BB was used at a concentration which had been previously used for in vitro work with monocytes (15 ng/ml, (Siegbahn 1990; Ernofsson 1996)), in contrast with the concentration of PDGF-BB in the serum-containing medium (851 pg/ml) as determined by ELISA analysis (3.2.3.1). In the serum-free milieu, neither Ang-2 nor PDGF-BB individually increased monocyte fibrinolysis (Figure 20, columns 2 and 3). Notably, the combination of both PDGF-BB and Ang-2 together restored the invasive action and resulted in a 3-fold increase in invasion above the untreated wells (Figure 20, column 4, p < 0.05).

Although Ang-2 and PDGF-BB administered together in the serum-free milieu induced a more pronounced increase in monocyte fibrin invasion than in a serum-containing milieu (for the same time period), it was noticed that the average number of cells accumulating in the bottom compartment was lower under serum-free conditions. Based on the results of the D-dimer ELISA discussed above (3.1.4.1), this was not surprising, as the control level of

![Figure 20](image-url)
monocye fibrinolysis was seen to be higher under serum-containing conditions. These data suggests that monocye invasion into fibrin is influenced by the combination of growth factors present in the serum.

These experiments strongly suggest that Ang-2 and PDGF-BB act cooperatively to influence monocye fibrin invasion. As seen from the serum-free investigations, neither growth factor individually affects monocye invasion, however together they cause a 200% increase in fibrin invasion above control cells. The comparison between invasion in the presence of serum and in serum-free conditions also suggests that the factors present in the serum may also play a role in affecting monocye invasion.

3.2.3.3 Analysis of the Chemotactic Potential of PDGF-BB

PDGF-BB has been satisfactorily used in a number of invasion and chemotaxis assays, and has been shown to exert a chemotactic effect on monotypes under certain in vitro conditions (Graves 1989; Siegbahn 1990; Ernöösson 1995). Other references, however, have contradicted the chemotactic effect of PDGF-BB (Graves 1989). Because of these conflicting results, the chemotactic potential of PDGF-BB was tested under the experimental conditions. Because none of the previous reports had detected any proliferative effect of PDGF-BB on monocytes, no proliferation assay was conducted.

The experimental model (see 2.2.3) chosen for these experiments was the same as the one described above (3.2.2.3), and has successfully been used in previous monocye chemotaxis studies (Siegbahn 1990; Ellingsen 2000).

In these experiments, monocytes were seeded onto the polycarbonate membrane, and incubated for 4 hours in serum-free medium with a PDGF-BB (15 ng/ml, (Siegbahn 1990)) concentration gradient. The experiments were quantified by counting the cells on the underside of the membrane (Ellingsen 2000). As described in 2.2.3, 5 representative fields from each of the triplicate experimental and control membranes were chosen at random under 10x magnification, yielding an average number of cells per field.
As can be seen, under the experimental conditions, PDGF-BB (Figure 21, $p > 0.05$) does not exert a chemotactic force on human peripheral blood monocytes. It was hypothesized that the movement of cells observed was once again due to random cell migration (chemokinesis), as the migration numbers were equivalent in the experimental and control membranes. While the lack of PDGF-BB-induced chemotaxis is in contradiction to the results of some other investigations (such as (Siegelman 1990)), it is possible that this difference could be accounted for by different experimental design such as different media used.

3.2.4 Summary

The use of a novel fibrin invasion assay system indicated that at concentrations of above 250 ng/ml Ang-2 increased monocyte invasiveness into fibrin clots in serum-containing environments. Further experiments were conducted using an Ang-2 concentration of 500 ng/ml, based on the data from the dilution curve. Under the experimental conditions, Ang-2 was not seen to stimulate either chemotaxis or proliferation in monocytes, so the choice of fibrin invasion assay was both valid and informative. In the presence of Ang-2, monocyte fibrin invasion increased 60% in a serum-containing environment.
The incorporation of the broad-spectrum inhibitors, aprotinin and GM6001, into the fibrin invasion provided evidence that the heightened fibrin invasion observed in the presence of Ang-2 was dependent on both serum proteases as well as MMPs. In addition, treatment of the fibrin invasion assays with cycloheximide stopped all cell invasion, indicating that the increased fibrinolysis in the presence of Ang-2 involved an upregulation of protein synthesis.

Upon removal of serum from the experimental set-up, it was noted that Ang-2 facilitates monocyte fibrin invasion in conjunction with PDGF-BB, and that individually the growth factors had no significant effect on monocyte fibrinolysis, suggesting that the two act as co-factors for increasing monocyte fibrinolysis. While both Ang-2 and PDGF-BB have previously been reported to interact with other growth factors in a cooperative manner (Maunoury-Pierre 1997; Halasz 1999; Padre 2002; Russo 2002), this is the first report of them acting together.

3.3 Analysis of Ang-2 and PDGF-BB Stimulated Monocyte Fibrinolysis

3.3.1 Introduction

The results from the inhibitor study (3.2.2.4) suggested that the Ang-2/PDGF-BB-mediated fibrin invasion was a result of upregulated fibrinolytic mechanisms. In this section several elements of both the plasminogen and the MMP fibrinolytic systems were analysed.

The capacity to secrete various compounds is an important property of cells in the monocyte-macrophage lineage, in addition to the phagocytic and antigen presenting functions (Peijer 2003). Monocytes are known to secrete a number of proteases in response to signaling molecules, including a number of different MMPs: MMP1, MMP2, MMP3, MMP8, MMP10, MT1-MMP, MT4-MMP, MMP19, MMP28 (Bar-Or 2003). Monocytes are also known to express elements of the plasminogen/plasmin system, including uPA and uPAR (Blasi 1997; Levi 2004), and human peripheral blood monocytes have previously been reported to produce up to 10^5 uPAR molecules per cell (Blasi 1987). As a multifunctional receptor, uPAR is known to promote pericellular proteolysis and matrix attachment, as well as affecting proteinase expression during macrophage differentiation (Mershikov 2004). uPA is produced at a lower rate than uPAR, but it has been observed that mice lacking uPA expression are not able to recruit sufficient numbers of macrophages during inflammation, suggesting that this enzyme is important in the cellular immune system (Peijer 2003).
The mechanisms of the monocotyledonous inversion could be inferred.

To examine the evolution of the monocotyledonous inversion, the size of these bands in the monocotyledonous

inversion were determined by monomers, secerns and trimers as well as....
3.3.2.1 Initial Analysis of Fibrin Degradation

FDP-containing conditioned medium samples were generated as discussed in 2.3.1.3. After 15 hours, the conditioned medium was drawn off and resolved on a 10% Fibrin gel, and FDPs detected using an anti-fibrinogen antibody. Bands 1 – 3 at 52, 37 and 14 kDa respectively were chosen as representative bands (as shown in Figure 22B) from the fibrin degradation pattern, and analyzed for 3 different donors in 4 independent experiments in triplicate, using the NIH-densometric scanning software (2.3.2.3).

![Image](image_url)

Figure 24. Treatment of monocytes with Ang-2 and PDGF-BB increases the level of fibrinolysis. A. All three bands (bands 1 – 2, 32, 37, 14 kDa, respectively) subjected to densometric analysis showed a significant increase in density in the presence of Ang-2 and PDGF-BB, which was not present during treatment with either PDGF-BB or Ang-2 alone. *p < 0.05, n = 4 experiments in triplicate 3 donors. B. Representative picture of monocyte fibrin degradation immunoblot. Arrows indicate the bands analyzed in A.

In all donors, a marked increase in FDP bands was seen in the immunoblots of the samples treated with Ang-2 and PDGF-BB, while the samples treated with either Ang-2 or PDGF-BB showed no significant increase (Figure 22A, group 4, p < 0.05). Samples treated with either growth factor alone did not show any significant alteration in the density of the FDP analyzed (Figure 22A, group 2 and 3). These observations further indicate that the increase in monocyte fibrin invasion is as a result of the increased fibrinolysis caused by the combination of Ang-2 and PDGF-BB, supporting the data obtained from the fibrin invasion and inhibitor studies (3.2).

Interestingly, the three bands analysed increased in density above the control samples at different levels - 38%, 73% and 50% respectively for bands 1 – 3. This suggested that Ang-2 and PDGF-BB increase fibrinolysis by upregulating certain elements of the fibrinolytic system, rather than causing a general increase in activity and proteolysis.
3.3.2.2 Further Analysis of Fibrin Degradation Patterns

As the FDP bands did not appear to increase at the same intensity in response to Ang-2/PDGF-BB treatment (3.3.2.1), it was possible that an analysis of the fibrin degradation pattern observed by immunoblotting might be able to shed some light on some of the fibrinolytins involved in the observed fibrin degradation.

<table>
<thead>
<tr>
<th>MMP-2</th>
<th>MMP-9</th>
<th>MT1-MMP</th>
<th>Plasmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>32</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
<tr>
<td>30</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
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<tr>
<td>28</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
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<tr>
<td>26</td>
<td>12.5</td>
<td>12.5</td>
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<td>24</td>
<td>12.5</td>
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<td>22</td>
<td>12.5</td>
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<td>20</td>
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<tr>
<td>18</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
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<tr>
<td>16</td>
<td>12.5</td>
<td>12.5</td>
<td>12.5</td>
</tr>
</tbody>
</table>

Table 12. Table showing the sizes of fibrin degradation products of cross-linked fibrin treated with fibrinolysins. All values are in kDa (Bini 1999). Unique bands are highlighted.

Table 12 above summarizes results obtained by Bini et al. (Bini 1999) in their analysis of the degradation of cross-linked fibrin by various fibrinolysins. Although the degradation patterns appear similar, there are unique bands in each degradation profile (highlighted), which might serve to identify the fibrinolytins involved in the degradation process.

In order to investigate the type of fibrin degradation bands produced by monocytes in response to treatment with Ang-2 and PDGF-BB, the immunoblots of the conditioned media used in 3.3.2.1 were reanalyzed, and all the bands sized and quantified using a molecular weight marker and densometric software.
Table 13. Analysis of fibrin degradation pattern from monocytes. Column 1 indicates the size of the fibrin degradation bands observed in monocyte-conditioned fibrinolysis as calculated from a molecular weight standard. Columns 2-5 indicate possible enzymes responsible for these bands.

<table>
<thead>
<tr>
<th>MW of Band</th>
<th>MMP-3</th>
<th>MMP-7</th>
<th>MT1-MMP</th>
<th>Plasmin</th>
</tr>
</thead>
<tbody>
<tr>
<td>88</td>
<td>✓</td>
<td></td>
<td></td>
<td>✓</td>
</tr>
<tr>
<td>84</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
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<tr>
<td>80</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td></td>
<td>✓</td>
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<td>✓</td>
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<tr>
<td>48</td>
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<td>✓</td>
<td></td>
</tr>
<tr>
<td>37</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
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<tr>
<td>30</td>
<td>✓</td>
<td>✓</td>
<td></td>
<td></td>
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<tr>
<td>24</td>
<td></td>
<td>✓</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>

The bands at 82 and 48 kDa are unique to the MT1-MMP degradation profile, strongly suggesting its involvement in the degradation of fibrin in the conditioned medium samples. Similarly, the bands at 84 and 58 kDa suggest the involvement of plasmin. As all the bands present that could have been caused by MMP-8 and MMP-7 degradation were also present in either the MT1-MMP or the plasmin degradation patterns, no conclusions could be deduced from the immunoblots for these two enzymes.

In order to extend these observations, the assay was repeated with the incorporation of plasminogen, apoprotein and GM6001. It was hoped that these additions would similarly provide information as to which fibrin degradation bands were a result of which enzymes.

Table 14. The presence of plasminogen, apoprotein and GM6001 alters monocyte fibrinolysis. Table represents the bands present in fibrinogen immunoblots of conditioned medium taken from monocytes grown on fibrin (column 1), with the addition of plasminogen (column 2), apoprotein (AP, 50 ng/ml, column 3) and GM6001 (0 µg/ml, column 4). ✓ indicates the presence of a band. N = 2.
Columns 3 and 4 (Table 14) represent the fibrin degradation patterns of monocytes seeded on fibrin when treated with either aprotinin or GM6001. The columns indicate that aprotinin is very effective in knocking out all plasmin activity, and also knocks out most MMP activity. This is probably due to the subsequent inactivation of plasmin-activated MMPs (van Hinsbergh 2006). Interestingly, the only band remaining in the aprotinin-treated samples was at 52 kDa (Figure 23) - one which is known to be generated by MT1-MMP fibrinolysis (Table 14). GM6001, on the other hand, selectively inhibited activity of certain MMPs, and many bands are present in the sample, albeit at much lower concentrations (Figure 23). In the product information sheet, GM6001 is reported to inhibit MMP1, 2, 3, 8, and 9. From the results presented above, however, it seems likely that GM6001 also inhibits MT1-MMP.

The addition of plasminogen to the fibrin gel (Figure 23, column 2) caused a general increase in proteolysis, further confirming that plasmin was integral to monocyte fibrinolysis. Interestingly, this upregulation was not uniform, and the bands at 56, 57, and 14 kDa were considerably darker than the other bands (Figure 23). This result, in contrast to a uniform upregulation of PDEs, does further suggest that the PDEs produced when monocytes are incubated with Ang-2 and PDGF-BB on fibrin are a result of both the plasminogen and the MMP systems.

While the results of this experiment are in no way conclusive, they do support the findings discussed in 3.2.2.1, that both serine proteases, such as plasminogen, and matrix metalloproteases, especially MT1-MMP, are involved in Ang-2/PDGF-BB-induced fibrinolysis. It was thought that the differences between the results of these experiments
and the invasion assays could be explained by differences in sensitivity of the assays, and also the possibility of the cells focusing their fibrinolytic activity in the invasion assays, and therefore invading the fibrin more quickly.

3.3.3 Elements of the Plasminogen System are Involved in the Upregulation of Fibrinolysis in Monocytes Treated with Ang-2 and PDGF-BB

As discussed in the section 1.2.1.1, the plasmin(ogen) fibrinolytic cascade consists of several serine proteases, including plasminogen, and the plasminogen activators tPA and uPA (Lijnen 2002; van Hinsbergh 2006). uPA activates plasminogen on the cell surface, where it is anchored by the receptor uPAR. Despite recent reports of cell surface-associated tPA activity (Grobmyer 1993; Ellis 1997), it is generally accepted that its major function is as a secreted plasminogen activator. As the results in 3.2.2.1 and 3.2.2 strongly suggest the involvement of plasmin in the observed monocyte fibrinolysis, the expression of tPA, tPA and uPAR were investigated in this section.

3.3.3.1 Analysis of RNA Expression

An analysis of tPA and uPA expression in monocytes by RT-PCR (section 2.6) revealed that under our experimental conditions uPA was expressed, but not upregulated, in monocytes treated with PDGF-BB and Ang-2 compared to untreated cells (Figure 24A). Conversely, tPA expression was not detected in any of the monocyte samples (lanes 1 - 4, Figure 24B), but was detected in the HSVEC sample (positive control, lane 6). When uPAR expression was analysed using RT-PCR (Figure 24C), the receptor was seen to be expressed in untreated monocytes (lane 1), as well as those treated with PDGF-BB (lane 2) or Ang-2 (lane 3). Interestingly, the expression of uPAR appeared to be marginally increased in monocytes treated with the combination of Ang-2 and PDGF-BB (lane 4).
Based on the PCR results, it was decided that further studies of the plasminogen system would be confined to uPA and uPAR.

3.3.3.2 Analysis of uPAR Protein Expression

A number of previous reports have identified both uPA and uPAR expression in monocytes (Pierleoni 2003; Levi 2004; Murdoch 2004). Unfortunately, at the time of this study, no commercially produced uPA antibodies could be sourced. Therefore, only uPAR expression could be investigated on a protein level. In order to confirm PCR results, uPAR was analyzed using cell lysates obtained from monocytes incubated with Ang-2 and/or PDGF-BB. Lysates were resolved on 10% SDS-PAGE gels, and uPAR concentration determined using immunoblotting.
Figure 25 presents a summary of an analysis of uPAR expression in 4 different donors in triplicate. As can be seen from the diagram, treatment of monocytes with Ang-2 and PDGF-BB together resulted in an 87% increase in the expression of uPAR on the cell membrane (Figure 25, column/lane 4). Treatment with PDGF-BB or Ang-2 alone did not significantly affect expression (Figure 25, column/lane 2 and 3). This suggests that the Ang-2/PDGF-BB-dependent monocyte invasion observed in 3.2 involved uPAR.

A survey of existing literature suggested that uPAR also functions as a secreted receptor when it is shed from the cell surface (Wilhelm 1999). In order to determine whether this receptor shedding was occurring under the experimental conditions, conditioned medium from monocytes treated with Ang-2 and/or PDGF-BB was also subjected to uPAR immunoblotting. No signal was detected in any of the experimental or control samples, strongly suggesting that the uPAR detected in the RT-PCR (3.3.3.1) remained immobilized on the cell surface until internalized and degraded.

3.3.3.3 Inhibition of uPAR Function

In order to link the observed upregulation of uPAR to the monocyte fibrin invasion, the serum-free fibrin invasion assay (2.2.7) was repeated with the incorporation of a uPAR function-blocking antibody (goat polyclonal, Abcam, Cambridge, ab3129). This antibody was
added to both the fibrin and the medium at a 4 μg/ml (manufacturer-recommended concentration).

As shown in figure 26 (p < 0.05), blocking uPAR activity in the fibrin invasion assay removed the increase in monocyte fibrinolysis seen during treatment with Ang-2 and PDGF-BB. Interestingly, however, the migration of the control monocytes treated with anti-uPAR were not significantly altered in comparison to the other control cells, indicating that uPAR was important in the Ang-2/PDGF-BB-mediated aspect of fibrinolysis.

It is interesting, however, that inhibition of uPAR did not cause fibrin invasion levels to drop below control levels. Recent research on invasion have suggested that both MT1-MMP and uPAR are co-expressed on the invadopodia of invading cells (Purmariak-Kazmierczak 2007). Furthermore, research on monocytes activated with E-LDL exhibited enhanced surface expression of uPAR and MT1-MMP, as well as increased secretion of MMP9 and uPA, while exhibiting enhanced invasion into Matrigel (May 2005). These observations contribute to the understanding that fibrinolysis in monocytes is potentially a complex interaction in which members of both the plasminogen/plasmin and the MMP fibrinolytic systems interact. It is therefore possible that inhibition of uPAR might affect MT1-MMP-dependent fibrinolysis.
3.3.3.4 Inhibition of uPA Function

Amiloride is a molecule composed of a guanidinium group containing a pyrazine derivative, and functions as a known uPA function inhibitor (Wang 1993; Jankun 2001; Dyer 2002). As recommended by the literature, amiloride was used at a concentration of 50 μM to inhibit uPA function in vitro. The LIVE/DEAD® Viability/Cytotoxicity Kit (2.2.4) and serum-free fibrin invasion assays (2.2.7) were repeated, with the incorporation of amiloride in both the fibrin gel and the culture medium.

![Graph showing fibrin invasion assay results](image)

Figure 27: uPA influences the action of Ang-2 and PDGF-BB in human monocyte fibrinolysis. The addition of amiloride to the fibrin invasion assay revealed the heightened fibrinolytic effect caused by Ang-2 and PDGF-BB (column 4).

*P < 0.005 against all other treatments.

3 experiments in triplicate (2 donors).

Treatment of the fibrin invasion assay with amiloride produced a very similar effect to that seen with anti-uPAR (Figure 27, p < 0.005). In both experiments, blocking either uPA or uPAR function resulted in the removal of the upregulated monocyte invasion seen during treatment with Ang-2 and PDGF-BB. In neither scenario, however, did the inhibition of function significantly lower the invasion below that of the control wells. This suggested that uPA/uPAR was integral to the upregulated fibrinolytic effect of Ang-2 and PDGF-BB.
3.3.4 Upregulation of MT1-MMP Expression in Monocytes Treated with Ang-2 and PDGF-BB

As discussed in the introduction (1.2.1.1), MT1-MMP is known to be a fibrinolytic MMP that plays a key role in the recruitment of monocytes during inflammation (Matins-Roman 2005). The analysis of PD patterns discussed in section 3.3.1 suggested its involvement in fibrinolytic effect observed through the stimulation of monocytes by Ang-2 and PDGF-BB.

3.3.4.1 Analysis of RNA Expression

An analysis of RNA isolated from monocytes treated with Ang-2, PDGF-BB, or both indicated that MT1-MMP expression in monocytes is regulated on a transcriptional level by the combination of Ang-2 and PDGF-BB.

![Figure 28](image)

As can be seen from the representative gel (Figure 28, lane 4), Ang-2 and PDGF-BB resulted in an upregulation of RNA copies in the cell. Neither Ang-2 nor PDGF-BB alone affected the expression of MT1-MMP (Figure 28, lanes 2 and 3). Because of the low quality of the PCR results, these results were confirmed by protein expression analysis.

3.3.4.2 Analysis of Protein Expression

As a membrane-bound MMP, MT1-MMP expression was analysed in the cell lysates of monocytes treated with Ang-2 and/or PDGF-BB. The results of 4 different donors analysed in triplicate are detailed in the figure 29 below.
Although MNG and XNLP are also known to be phosphorylated (L.2, 7), there is little in the

2.3.1 Analysis of MNG and XNLP expression

not yielded any clear results.

The observed changes in the level of protein expression, however, are not dependent on the type of cell line or the treatment conditions, indicating that the observed changes are not due to differences in the conditions used. The observed changes in MNG and XNLP expression are not due to differences in the conditions used.

Although MNG and XNLP are also known to be phosphorylated (L.2, 7), there is little in the

2.3.1 Analysis of MNG and XNLP expression

not yielded any clear results.
3.3.5.1 Analysis of RNA Expression

Reverse transcription analysis of MMP3 and MMP7 expression in monocytes treated with Ang-2 and PDGF-BB did not reveal significant expression of either MMP in any of the different samples. This suggested that monocytes under the conditions studied did not secrete either of these MMPs.

3.3.5.2 Analysis of Protein Expression

In order to confirm the gene expression results above (3.3.5.1), MMP2 and MMP7 protein expression was also studied. While unlikely, it is possible for RNA analyses not to correlate with protein expression due to translational and post-translational mechanisms through increases in mRNA half-lives, and intracellular storage pools. As both MMP3 and MMP7 are secreted MMPs, the conditioned medium from treated monocytes was analysed using immunoblotting for MMP3 (57 kDa) and MMP7 (28 kDa).

Both the MMP3 and MMP7 immunobLOTS revealed similar bands at 30 kDa. This signal was also present in immunobLOTS detected with the secondary antibodies only, proving that it was a false positive. This signal had also been observed during unrelated research done by other members of the laboratory, and it was probable that this band was due to non-specific binding of the antibodies to BSA present in the serum-free medium (personal communication, Mrs Mona Bracher, CVRU, UCT).

Neither the MMP3 nor the MMP7 immunobLOTS had any other bands, and it was decided that MMP3 and MMP7 were either absent from the samples, or expressed in very low quantities.

3.3.5.3 Analysis of Protein Expression Using Zymography

Zymography is an alternative method for the detection of MMPs, which was used to reanalyse the samples from 3.3.5.2. It is an electrophoretic technique for the identification of proteolytic activity in enzymes separated in polyacrylamide gels co-polymerised with a protein substrate under non-reducing conditions. Analysis is based on the ability of MMPs to refold after denaturing SDS is removed, and to digest the co-polymerized substrate. Staining the gel with Coomassie Blue allows the digested areas to be visible as a clear band against a uniform blue background. While the protocol allows both the active and zymogen forms of
the MMPs to digest the substrate, the size difference between two forms allows for quantification of active/pro-forms of the MMPs in the sample.

As both gelatin and casein are substrates of MMP3 and MMP7, zymographic analysis of expression in conditioned medium was conducted using both as substrates. Neither gelatin nor casein zymography detected any significant expression of either MMP. These results, together with those from 3.3.4.1 and 3.3.4.2 strongly indicated the lack of involvement of MMP3 and MMP7 in the monocyte fibrinolysis observed in these experiments.

3.3.6 Summary

The Ang-2/PDGF-BB-stimulated fibrin invasion described in 3.2 was confirmed to be as a result of increased fibrinolysis through immunoblotting analysis of PAP. Furthermore, an analysis of the specific bands in the fibrinogen immunoblot indicated that both the plasminogen and the MMP fibrinolytic systems were involved, as had previously been observed in 3.2.2.5.

Analysis of various elements of both fibrinolytic systems showed that uPAR and MT1-MMP expression were upregulated on the RNA and protein levels. Furthermore, function inhibition of uPA and uPAR confirmed the critical involvement of these fibrinolytic elements in the Ang-2/PDGF-BB response. Interestingly, uPA secretion is a characteristic feature of activated monocytes (Pejler 2003), suggesting that the upregulation of the uPA/uPAR system is in line with heightened monocyte activity.

tPA, MMP3 and MMP7 expression, however, was not detected using any of the available methodology. These data suggested that the observed upregulation of fibrinolysis in monocytes involved specific elements of both fibrinolytic systems, and not an overall increase in proteolytic mechanisms.

3.4 Regulation of MMP9 and MMP2 and Monocyte Activation

3.4.1 Introduction

MMP9 (as discussed in 1.2.1.2.2) has been suggested as a component of the angiogenic switch, through its involvement in VEGF-A release from the extracellular reservoir (Bergers 2000). These data suggest a pivotal role for the expression of MMP9 in wound healing. In
addition, both MMP2 and MMP9 have also previously been shown to affect leukocyte trafficking during inflammation (McQuibban 2000; Wetzler 2000; Corry 2004; Greenlee 2006). Thus increased expression of gelatinases in monocytes could suggest a more invasive profile for the leukocytes involved in wound healing. Furthermore, MMP2 and MMP9 have previously been described as markers for monocyte activation and differentiation (Xie 1998; Menshikov 2004). Therefore, sustained upregulation of their expression would suggest that Ang-2 and PDGF-BB were activating agents for monocytes.

MMP9 was first described because of its ability to cleave ECM molecules such as collagens and gelatine in vitro. Subsequently the list of its substrates has expanded to include a number of other matrix and non-matrix compounds (Lelought 2001). Interestingly, a recent nephropathy study showed MMP9 able to directly degrade fibrin both in vitro and in vivo (Lelought 2001), cleaving the same fibrin and fibrinogen chains as MT1-MMP (Hiraoka 1998). In endothelial cells, MMP2 has been seen to activate pro-uPA bound to uPAR, thus initiating fibrinolytic activity in a VEGF-modulated sequence of events (Prager 2004). Consistent with these observations, a recent study in MMP2/MMP9 double null mice showed high levels of fibrinogen accumulation, suggesting that the absence of both gelatinases impairs fibrinolytic activity (Lambert 2003).

Furthermore, MMP2 is connected to fibrinolysis, in that its activation is dependent on another fibrinolytic MMP, MT1-MMP. MMP2 forms a trimolecular complex with MT1-MMP and TIMP2 on the cell surface, where it is activated by proteolytic cleavage. The activation of MMP2 is therefore directly related to the expression and activity of MT1-MMP (Kazes 2000; Lafleur 2001).

MMP2 and MMP9 expression and activity were analysed using gelatin zymography. This methodology involves the degradation of the gelatin substrate by the gelatinases in the samples. Prior to quantification analyses it was necessary to determine the range in which the concentration of enzyme was reflected on the gel in a linear manner. The band intensity was determined to lie in the linear region by the dilution curve using a positive control for MMP2/MMP9, which was a kind gift from Mrs Merv Brucher (CVRU, UCT). Gels were also run with a negative control (cell-free medium) to ensure that the growth medium had no latent gelatinolytic activity.
3.4.2 Ang-2 and PDGF-BB Affect MMP9 Expression

Monocyte production of MMP9 has been previously well documented (such as Shapiro 1993; Levi 2004; Murdoch 2004). Interestingly, in addition to being a marker for monocyte activation (Xie 1988; Menshikov 2004), the level of expression has been shown to be dependent on a number of different factors, such as substrate and the presence of other factors (Lepidi 2001). For example, Galt et al demonstrated that MMP9 expression increased synergistically following adhesion to collagen and platelets (Galt 2001).

3.4.2.1 Analysis of RNA Expression

Reverse transcription analysis revealed that monocytes treated with Ang-2 and PDGF-BB exhibited increased MMP9 expression in comparison to untreated cells, or those treated with either Ang-2 or PDGF-BB (Figure 30, lane 4).

![Figure 30. Ang-2 and PDGF-BB together increase monocyte MMP9 RNA expression. Top panel is of MMP9 RT-PCR (129 bp), and bottom is of vimentin (212 bp) RT-PCR on the same samples. Lane 1 is of untreated cells, 2 of Ang-2-treated cells, 3 of PDGF-BB-treated cells, and 4 of Ang-2 and PDGF-BB-treated cells.]

3.4.2.2 Analysis of Protein Using Gelatin Zymography

MMP9 is secreted by monocyte in the zymogen form, and activated in the extracellular environment. Using gelatin zymography, MMP9 expression and activation was analysed under a number of different conditions detailed below. Verification of the MMP activity observed in the conditioned medium was done by incubating zymogram gels in a substrate buffer containing the calcium-chelating agent EDTA. MMPs are dependent on calcium for their structural integrity and one of the features that distinguishes MMPs is the inhibition of their activity by chelating agents such as EDTA (Lepidi 2001). Two gels were run containing identical samples of cell-conditioned medium. One gel was incubated in calcium containing substrate buffer, and the other in three concentrations of EDTA in substrate buffer (25, 50, 100 mM). Inhibition of gelatinolytic activity by EDTA confirmed the proteolytic enzymes under analyses were MMPs (data not shown).
MMP expression of mouse lung was determined in two separate experiments involving wild-type and Pp5F6 deficient mice. The results showed that MMP expression levels in wild-type mice were significantly higher than in Pp5F6 deficient mice. Additionally, the results indicated that MMP expression was not significantly affected by Pp5F6 deficiency in the lungs of mice.
and that Ang-2 and PDGF-BB simply accelerate this process. In both treated and untreated samples, the gradual increase in active MMP9 (82kDa) was very similar (Figure 31B, gel insert).

### 3.4.3 Analysis of MMP2 Expression

Although opinions remain divided regarding the extent of MMP2 expression by monocytes, significant MMP2 expression has been reported in a number of different papers, such as (Bar-Or 2003). As MMP2 expression is known to be upregulated during monocyte differentiation and activation, it is possible that differing methodologies of monocyte isolation could influence MMP2 expression.

#### 3.4.3.1 Analysis of RNA expression

![Monocyte RNA expression analysis](image)

Monocytes isolated by density centrifugation and fibronectin adhesion (2.1) did secrete MMP2 (Figure 32, lane 1). Treatment with PDGF-BB and/or Ang-2 did not significantly affect MMP2 production (Figure 32). Because of the relatively low quality of the PCR, this result was confirmed by gelatin zymography.

#### 3.4.3.2 Analysis of Protein Expression in Conditioned Medium

As both MMP2 and MMP9 have gelatinolytic activity, MMP2 expression was also analysed using gelatin zymography, as it was observed that in the conditioned medium of most donors, MMP2 activity was detectable (Figure 33).

In contrast to the highly regulated MMP9 expression, MMP2 expression was not significantly altered by the addition of Ang-2, PDGF-BB, or a combination of both growth factors. Interestingly, however, all MMP2 detected was in the 82 kDa active form, in contrast to the MMP9 zymogen expression.
3.4.3.3 Analysis of Protein Expression in Cell Lysates

MMP2 activation has been shown to be mediated by complex formation with MT1-MMP and TIMP2 on the cell membrane (Kazes 2009). Therefore, because of the upregulation of MT1-MMP discussed in 3.3.4, it was thought possible that more MMP2 could be bound to the cell surface in activation complexes with MT1-MMP. Cell lysates prepared as detailed in 2.3.1.4 were analysed using gelatin zymography (2.3.3.1).

No MMP2 expression was detected in the cell lysates analysed using gelatin zymography. The lanes in which the cell lysates were run exhibited high levels of streaking and background, which suggested that the technique was not suited for analyzing cell lysates, and no conclusions could be drawn from these experiments.

3.4.4 Summary

MMP9 expression in monocytes was seen to be upregulated in the presence of Ang-2 and PDGF-BB, and the expression of the 92kDa zymogen form was significantly increased in treated samples for up to 24 hours. After 24 hours the upregulation in response to the
growth factors was lost, suggesting either that the MMP9 upregulation seen in response to Ang-2 and PDGF-BB was not as a result of increased monocyte activation, or that MMP9 expression reaches a plateau in differentiating monocytes which Ang-2 and PDGF-BB accelerate.

MMP2 expression was not influenced by Ang-2 and PDGF-BB expression, although contrary to some reports in the literature, MMP2 expression by monocytes was noted in most donors. Despite upregulation of MT1-MMP expression (3.3.4), no changes in MMP2 expression were noted. No other gelatinase expression, such as MMP12 (54kDa), was detected using zymography.

3.5 Analysis of Possible Receptors for Ang-2 and PDGF-BB Signaling

3.5.1 Introduction

When Ang-2 was first discovered, it was thought to be a growth factor restricted to signaling through the endothelial cell-specific receptor Tie-2 (Mainiopierre 1997). Increasingly, recent reports have linked Tie-2 expression to other cell types (as discussed in 1.4.2), as well as suggesting that Ang-2 is able to signal in cells lacking the Tie-2 receptor.

A number of independent investigations have reported Tie-2 expression on non-endothelial cells, including neutrophils (Lemieux 2005), mural precursor cells (Juularo 2003), and endothelial progenitor cells (EPCs) (Sato 1995). Indeed, Shahara et al. (Shahrara 2002) described, using immunostaining, that Tie-2 was expressed in synovial tissue lining cells, macrophages, and SMC in both arthritic and control patients. Other contradictory reports, however, propose opposing results, reporting no Tie-2 expression on neutrophils (Fleißer 2006). These studies suggest that Tie-2 expression is not yet fully understood.

Recently, Ang-2 has been showed to be able to signal in the absence of Tie-2. Fibroblasts have been shown to adhere to Ang-2 through integrin α6 (Carlson 2001), while cardiac and skeletal myocytes adhere through integrins αv, α6, and β1 (Dallabrida 2005). Ang-2 has also been implicated in tumour progression, where tumour cells lack Tie-2 expression (Etoh 2001; Guo 2005; Liu 2006) (see section 1.4.24). These studies indicate that Ang-2, through integrin signaling, has an effect on a much broader range of cells than was previously anticipated.
Integrin-mediated signals play an important role in regulating many leukocyte functions (Yurochko 1992, Reyes-Reyes 2002). In monocytes and macrophages, the β subfamily are involved in cell-cell interactions, cell migration and phagocytosis. The δ subfamily, on the other hand, control adhesion to extracellular matrix proteins, such as fibronectin and the activation of genes involved in inflammation (Reyes-Reyes 2002; Clark 2003). When bound to their natural ligands, both types of integrins activate many signaling pathways, including Ras, MAP kinase, FAK, Src, Rac, Rho, and PI-3-K (Eliceiri 2001). In addition, many recent investigations have demonstrated that cell adhesion receptors, such as integrins, and growth factor receptors act cooperatively in determining cell signaling (Eliceiri 2001). The receptors known to interact with integrins include PDGFR-β and uPAR (Plapper 1995; Sandberg 1996; Pluskota 2003; Pluskota 2004).

PDGFR-β expression has been noted on monocytes during the differentiation into macrophages, with low expression during the early activation (Krettek 2001), and a strong signal in fully-differentiated macrophages (Inaba 1993; Kumatagi 2002), while PDGF-BB action on monocytes has previously been documented (Singhahn 1990; Runnsson 1996).

PDGF-BB/PDGFR-β have previously been shown to interact with a number of different integrins (Schneller 1997), such as integrin β1 and αβ3. Ahlem et al showed that in fibroblasts, PDGF-BB modulates the membrane mobility of β integrins, thereby modulating a migratory response, which includes the reorganization of the actin cytoskeleton in a PI-3-K and protein kinase C (PKC)-dependent manner (Ahlem 2004). PDGF-BB has also been shown to stimulate β1-integrin-mediated adhesion and spreading in SMC, in a PKC-dependent manner (Kappert 2000). PDGFR-β has also been seen to associate with various integrins, including αβ2, and the engagement of the integrin in cell-matrix interactions has been seen to increase the response of the growth factor receptors to their ligands (Schneller 1997).

The experiments in this section provide an initial analysis of the receptors used by Ang-2 and PDGF-BB to signal in monocytes.

3.5.2 Analysis of Tie-2 Expression in Entire Isolated Monocyte Population

While Tie-2 expression has recently been reported on a number of different cell types (Koh 2002; Lemieux 2005), Tie-2 expression on monocytes is still an issue under discussion. Recently there have been suggestions that Tie-2 is expressed on a minority of blood monocytes (resident macrophages CD14+CD16−) (Venneri 2007), representing a specifically
pro-angiogenic subpopulation of circulating monocytes. There have also been reports of circulating EPC expressing Tie-2 (Anghelina 2002), although there is no definitive opinion on what EPCs are – poorly differentiated endothelial precursors of angioblast type, or similar to adult monocytes with the ability to transdifferentiate.

Tie-2 expression by monocytes was investigated under the experimental conditions using both RT-PCR and flow cytometry.

When the expression of Tie-2 on monocytes was analysed by RT-PCR (as detailed in 2.4), no significant Tie-2 expression was noted in the whole monocyte population under the experimental conditions. Figure 34A is a representative gel of the RT-PCR results. The top picture is of the Tie-2 PCR, and the bottom of the PCR of the HPRT housekeeping control gene. As can be seen, neither untreated monocytes (Figure 34A, lane 1), nor monocytes treated with PDGF-BB (Figure 34A, lane 2), Ang-2 (Figure 34A, lane 3) or a combination of both growth factors (Figure 34A, lane 4) showed any Tie-2 expression. In order to confirm these negative results, fibroblasts were used as a negative control (Figure 34A, lane 5), and HSVEC as a positive control (Figure 34A, lane 6) (Maisenbürger 1997; Yu 2001).
As it is possible that mRNA expression may not accurately reflect the steady state protein levels, Tie-2 expression in monocytes was also analysed using Tie-2 flow cytometry. Once again, HsVEC were used as a positive control, while human dermal fibroblasts were used as a negative control. Figure represents an average plot from three independent experiments (3 donors). As can be seen, while 95% of endothelial cells were positive for Tie-2 (Figure 34B, black line), monocytes (Figure 34B, dashed line) and fibroblasts (Figure 34B, grey line) displayed no discernable expression. These results, together with those obtained using RT-PCR, suggest that the monocytes isolated by density centrifugation in these experiments were a Tie-2 negative cell population.

Although these findings are in contradiction with those of Venneri et al. (Venneri 2007), it is possible that the dissimilar isolation procedure could account for these differences. In their study, Venneri et al. used magnetic beads to positively and negatively select for certain cell populations. These enrichment procedures therefore might have been able to select the Tie-2+ monocytes, which may have been lost during the isolation procedure described in 2.1.1. Furthermore, the analyses of uPAR, MT1-MMP and MMP9 expression in response to Ang-2 and PDGF-BB described above (Section 3.3) strongly suggest that the number of cells necessary to produce the relative increases in protein expression observed would necessitate a larger percentage of the population to be activated than the percentage of Tie-2-positive cells described by Venneri.

### 3.5.3 Analysis of PDGFR-β Expression

As described in section 1.4.1.1, the PDGF-BB ligand is able to bind to all three PDGF receptors (PDGFRα, PDGFRβ, and PDGFRγ) which differ in function mainly through their expression patterns (Funa 2003). As PDGFRβ expression was previously reported on monocytes in vivo (Kumagai 2001), it was selected for further investigation.

#### 3.5.3.1 Analysis of RNA Expression

An analysis of mRNA extracted from monocytes treated with Ang-2 and PDGF-BB revealed that PDGFR-β expression was upregulated in the presence of Ang-2 and PDGF-BB, and also moderately in the presence of Ang-2 alone (Figure 36). Cells treated with PDGF-BB alone, as well as untreated cells exhibited only very faint PDGFR-β signals.
This result suggests not only that PDGFR-β is instrumental in PDGF-BB signaling, but also that Ang-2 is instrumental in facilitating PDGF-BB signaling by upregulating the PDGF receptor on monocytes.

3.5.3.2 Analysis of PDGFR-β Protein Expression

The upregulation in PDGFR-β mRNA expression reported in 3.5.3.1 was compared to protein expression using a PDGFR-β ELISA kit obtained from R&D Systems as described in 2.3.5. Cell lysates (2.3.5) taken at 15 hours from cells treated with Ang-2 (500 ng/ml) or PDGF-BB (20 ng/ml) were used for this ELISA.

The results displayed below further suggest that the presence of Ang-2 in vitro causes an upregulation of monocyte expression of PDGFR-β (Figure 37, columns 3 and 4), as suggested by the RT-PCR analysis. Upon the addition of Ang-2, (in the presence or absence of PDGF-BB) a low, but detectable PDGFR-β signal was clearly observed in the cell lysate samples. Unlike the PCR analysis (2.5.3.1), however, the level of PDGFR-β expression was seen to be much the same between the Ang-2-treated cells, and those treated with Ang-2 and PDGF-BB.
Interestingly, however, untreated monocytes, or those treated with PDGF-BB (Figure 50, column 1 and 2) did not display discernable PDGFR β expression. This suggested that part of the mechanism in which Ang-2 works on monocytes is to enhance the expression of PDGFR β, thereby facilitating PDGF-BB signaling otherwise absent in untreated cells.

3.5.4 Analysis of Alternative Signaling Molecules for Ang-2

3.5.4.1 Analysis of Selected Integrin Expression on Monocytes Using Immunocytochemistry (ICC)

In the absence of detectable Tie-2 expression in the monocyte populations, it was probable that Ang-2 was signaling through another type of receptor. An increasing number of reports suggest that Ang-2 is able to interact with cells in the absence of Tie-2, through alternative receptors such as integrins (Carlson 2001; Jones 2003; Ballabriga 2005). In order to investigate the possibility that Ang-2 was interacting with monocytes through a member of the Integrin family, a preliminary study of integrin expression was conducted using the Integrin Classics Investigator Kit (Chemicon International, Temecula, ECM435). This kit provided antibodies to a number of different integrins, including α5, β1, β2, αβ3, αδβ1, αγβ1.
LabTek Permanox chamber slides were used for growing the cells, thereby circumventing the necessity of coating the slides with a protein such as fibronectin, as is necessary for glass slides. Furthermore, the removable chambers allowed the cells to be incubated with Ang-2 and PDGF-BB directly on the slide. After the incubation, the cells were fixed with ice-cold methanol and stained with the antibodies from the Integrin Classics kit as described in 2.5.1.1.

Of all the antibodies used, only the antibody to integrin β2 (Figure 37) gave a convincing signal in all cell treatments. The antibodies to integrins α5, (Representative shown in figure 38) and β1 gave very low signals, while the other two antibodies showed no signal at all.

The pictures obtained by fluorescent microscopy suggested that the β3 integrin was localized on the cell surface around the borders of attachment to the slide. In order to further investigate this observation, the slides were re-examined using confocal microscopy. Unlike regular microscopy techniques, only light from the focused point contributes to the final image in a confocal system, which allows high-resolution pictures from a well-defined optical section to be produced. Using a 63x water-immersion lens on the confocal microscope, the slides were optically sectioned at 1.5 μm per section, resulting in 10 sections per field of vision.

Figures 29 and 40 are representative optical stacks taken of monocytes. Figure 29 shows untreated monocytes, while figure 40 shows a cell stimulated with Ang-2 and PDGF-BB. As can be clearly seen both optical stacks, the integrin β3 is clearly localized on the cell surface, and is associated with focal adhesions on the interface between the cellular membrane and the growth surface and in the membranous projections (Figures 29 and 40, clear arrows). It was also noted that a signal was obtained in the perinuclear region (Figures 29 and 40, dotted arrows). By analyzing the optical sections, it was decided that this signal was perinuclear, and a by-product of the permeabilization of the cell membrane during sample preparation (Personal communication, Mrs Liz van der Merwe, Dept Anatomy & Cell Biology, UCT, RSA).

Reports in the literature suggest that some growth factors alter the membrane distribution of integrins on cell surfaces, thus altering the signaling of the integrins. PDGF-BB, for example, has previously been observed to modulate mobility of β3 integrins in fibroblasts (Ahlen 2004), as well as with β3 integrins (Grundstrom 2003). Interestingly, however, in a
study of human monocytes Maties-Roman et al (Matias-Roman 2005) suggested that the αvβ3 and αvβ6 dependent upregulation of MT1-MMP was more dependent on integrin receptor occupancy, rather than aggregation.

Although it was initially hoped that any possible changes in regulation or distribution could be analysed using microscopy, no changes in distribution were detected using the microscopy facilities available. Alternative methods were therefore utilized in order to discern the possible association between Ang-2 and integrin β3.

3.5.4.2 Inhibition of Candidate Integrin Functioning

Due to the levels of expression seen through ICC, it seemed likely that integrin β3 was a candidate for Ang-2 signalling. This hypothesis was tested by adapting the serum-free fibrin invasion assay (2.2.7) so as to include a β2 integrin function-inhibiting antibody (Chemicon, Temecula, MAB19602Z) in both the fibrin and conditioned medium.

Figure 41: Functional inhibition of integrin β3 caused loss of Ang-2/PDGFrBB-dependent fibrinolytic. Addition of function-inhibiting and human β3 integrin (column 4) caused the Ang-2/PDGFrBB-dependent increase in monocyte fibrin invasion (column 2) to drop to control levels (column 1). In the absence of Ang-2 and PDGF-BB the antibody did not significantly affect baseline monocyte fibrin invasion (column 3). *P < 0.05, n = 2 experiments in triplicate (2 donors).

As can be seen from figure 41 above, the functional inhibition of the β3 integrin family removed the upregulation of fibrin invasion caused by Ang-2 and PDGF-BB. Interestingly, the inhibition profile was very similar to that obtained during uPAR inhibition.
Figure 37. Integrin β3 expression as detected by Immunohistochemistry.

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Figure 38. Integrin αβ3 expression in macrophages under experimental conditions.

αβ3
Figure 38. Optical Z-stack through two monocytes. Images are numbered from cell surface (1) to culture plate (11).
Figure 40. Optical Z-stack of monocytes treated with Ang-2 and PDGF-AB. Images are numbered from cell surface (1) to culture plate (7).
3.5.4.3 Immunoprecipitation of Ang-2 with Integrin β2

In order to further test the hypothesis of integrin β2 as the receptor for Ang-2 in monocytes, cell lysates from cells treated with Ang-2 were subjected to immunoprecipitation with an antibody for integrin β2 (Abcam, Cambridge, ab657). As detailed in section 3.5.2, cell lysates were mixed with the monoclonal antibody for human integrin β2, and the antibody precipitated using Sepharose G beads. Following precipitation, the supernatants were analysed by SDS-PAGE (2.3.3) and detected for the presence of Ang-2 using a polyclonal antibody (2.3.2).

Figure 42 below shows a representative immunoblot of the immunoprecipitation results. The antibody for integrin β2 did indeed co-precipitate with the recombinant Ang-2 in monocyte cell lysates, as shown by a band (Figure 42A, lane 1) corresponding in size (66 kDa) to the Ang-2 standard (Figure 42A, lane 4). This Ang-2 band was absent in samples treated with either integrin β2 antibody and sepharose G beads (Figure 42A, lane 2), or with Ang-2 and sepharose G beads (Figure 42A, lane 3).

Although the band at 66 kDa in the sample (Figure 42, lane 1) was relatively low in signal, it was the only band present in the sample, and corresponded to the Ang-2 standard (Figure 42, lane 4). Furthermore, two immunoprecipitation controls were run to ensure that the result was valid. Samples of cell lysates from cells cultured without Ang-2 and immunoprecipitated (Figure 42, lane 2) did not have a band at 66 kDa, suggesting that the antibody was not detecting another cell lysate protein. Furthermore, samples of cell lysates from cells cultured with Ang-2, but not treated with sepharose G beads (Figure 42, lane 3)
also did not have a band at 66 kDa, suggesting that Ang-2 only precipitated in the immunoprecipitation protocol in the presence of sepharose G beads.

When the blot was stripped and re-detected for integrin β3 (85 kDa), a band was present in the two samples to which the β3 antibody was added (Figure 42B, lanes 1 and 2). This confirmed that the sepharose G beads were pulling down the antibody together with its ligand. This result, together with the inhibition study reported above (3.5.1.2) strongly suggests that in human monocytes, Ang-2 signals by binding to integrin β3. Due to imperfect antibody stripping during the rebinding of the membrane, the Ang-2 standard visible in Figure 42A (lane 4), was still visible in Figure 42B (lane 4), and the signal of the integrin β3 was lower than it would have been on a new blot.

3.5.5 Summary

In this section the possible receptors for Ang-2 and PDGF-BB on monocytes were investigated. The analysis of Tie-2 expression on monocytes by RT-PCR and flow cytometry revealed that under experimental conditions, monocytes did not express this receptor. While these results are in contradiction to other recent reports (Vernieri 2007), it is possible this could be accounted for by differing monocyte isolation methodology. Furthermore, it seemed unlikely that the large increases in the expression of various proteins in response to treatment with Ang-2 and PDGF-BB could be accounted for by the percentage of Tie-2 positive monocytes (in relation to the whole population) previously reported. These data further confirm that Ang-2 is not restricted to Tie-2 signalling.

Ang-2 has recently been shown to signal through a number of different integrins, on a variety of different cell types (Carlson 2001; Emb 2001; Daliabkda 2005; Guo 2005; Hu 2006). The expression of a number of different integrins was investigated on monocytes under experimental conditions. Immunohistochemical analyses revealed a very high signal for integrin β3, a known leukocyte-specific integrin (Din 2000). When the function of this integrin was inhibited in monocytes, it was observed that the heightened fibrin invasiveness seen in response to Ang-2 and PDGF-BB was lost, while the fibrin invasion of control cells treated with the antibody was unaffected. When an immunoprecipitation of integrin β3 was performed, Ang-2 was detected in the pull-down product, confirming that Ang-2 binds to the integrin. These novel data strongly suggest that Ang-2 signals through integrin β3 in human monocytes.
PDGFR-β expression was investigated using RT-PCR and ELISA methodologies. In both investigations it was observed that Ang-2 upregulated the expression of PDGFR-β, while PDGF-BB had no effect. Interestingly, while the RT-PCR suggested a very low baseline level of PDGFR-β expression in untreated and PDGF-BB-treated cells, results from the ELISA indicated an expression of PDGFR-β protein on these cells. This suggests that one of the mechanisms through which Ang-2 interacts with PDGF-BB is to enhance (or possibly initiate) PDGFR-β transcription and translation.

3.6 Investigation into the Nature of Ang-2/Integrin β2: Signaling

3.6.1 Introduction

Members of the β2 integrin family are the dominating integrins expressed on leukocytes, and they play a major role in leukocyte cell-cell and cell-matrix adhesions during inflammation and other immune responses (Arnaout 1990; Dib 2000). They also contribute to chemotaxis, adhesion to cytokine-activated endothelium and transendothelial migration of monocytes. β2 integrins are signaling receptors, but they are also targets of, and are functionally affected by, intracellular signals. Accordingly, it is generally accepted that when discussing signaling, two types of signaling by β2 integrins should be recognized: transmission of signals into the cell following binding of ligands or counter-receptors to the integrins ("outside-in", such as uPAR (Simon 1996)), and regulation of the avidity and conformation of integrins by signals generated by other receptors within the cell ("inside-out"), such as activating signals.

One of the initial events in β2 integrin mediated signaling is the activation of various non-receptor tyrosine kinases, such as members of the src and FAK families. The precise mechanisms by which β2 integrins initiate the activation of these tyrosine kinases, however, are unknown (Dib 2000). Engagement of the β2 integrins on leukocytes is also associated with a significant alteration of the cellular cytoskeleton, leading to cell spreading and locomotion. This involves the activation of the small GTP-binding proteins Ras and RhoA (Dib 2000; Laudomia 2002).

PDGF and PDGFR have previously been reported to interact with integrins on the cell surface. For example, in fibroblasts, in cooperation with integrins, PDGF-BB were seen to produce a marked, transient activation of the ERK class of MAPK (Miyamoto 1996). Furthermore, the growth factor receptor was itself induced to aggregate transiently by
integrin-coated beads in a process requiring both aggregation and occupancy of integrin receptors, suggesting synergism between integrins and growth factors triggering tyrosine phosphorylation of PDGF receptors (Miyamoto 1996; Sundberg 1996). It was also suggested that integrin-growth factor synergy might operate through the downstream effects of integrins and of occupied growth factor receptors to be additive or synergistic (Miyamoto 1996).

In this section the possible association of the Ang-2-associated integrin $\beta_3$ with uPAR was investigated. Furthermore, the upregulation of proteases observed in response to treatment with Ang-2 and PDGF-BB was investigated with regards to integrin $\beta_3$ inhibition. Finally, attempts were made to identify a signaling pathway through which Ang-2 and PDGF-BB were upregulating monocyte fibrinolysis.

3.6.2 Immunoprecipitation of uPAR with Integrin $\beta_3$

Integrin $\alpha_{\text{IIb}}$ and uPAR have previously been shown to form a functional unit on monocyte cells (Simon 1996). Together they have been shown to mediate complementary functions and promote the degradation of fibrin(ogen), as well as conferring adhesive properties on the cells (Simon 1996; Silvin 1996). As uPAR expression was seen to be upregulated in monocytes treated with Ang-2 and PDGF-BB (3.3.3.2), it was decided that the immunoprecipitates obtained above (3.5.4.3) would be used to investigate the level of uPAR association with integrin $\beta_3$ in the presence of Ang-2.

![Image](image.png)

**Figure 43: uPAR co-immunoprecipitated with integrin $\beta_3$**

A. uPAR immunoblot. B. Integrin $\beta_3$ immunoblot. Lane 1 represents Ang-2-containing cell lysate treated with integrin $\beta_3$ antibody and sepharose G beads. Lane 2 represents samples containing Ang-2 and challenged with sepharose G beads. Lane 3 represents sample without Ang-2 treated with integrin $\beta_3$ antibody and sepharose G beads. n=3.

As can be seen in the representative figure (Figure 43), in both samples treated with the antibody to integrin $\beta_3$, uPAR expression was detected by uPAR immunoblotting. Interestingly, in all the samples analyzed (n = 3), the uPAR signal in the sample treated with Ang-2 (lane 1) was significantly stronger than in the untreated sample (lane 3), while the
levels of integrin \( \beta_2 \) expression were equal in both samples. This suggested that the upregulation of uPAR protein expression (3.5.3.2) was accompanied by the increased association of uPAR with integrin \( \beta_2 \). This coupling also suggested a functional association between the two receptors.

3.6.3 MMP9 Expression in Response to uPAR Inhibition

Pro-MMP9 expression in the THP-1 monocyte cell line had previously been shown to be dependent on uPA/uPAR expression (Menshikov 2004). It was therefore possible that the Ang-2/PDGF-BB-dependent upregulation of MMP9 would be mediated by uPAR. Indeed, the association of uPAR with integrin \( \beta_2 \) described above (3.5.5.1), as well as the Ang-2-mediated upregulation of this association, strongly suggests a functional link between the two receptors.

The expression of MMP9 was analysed in the conditioned medium of cells treated with the function-blocking uPAR antibody (see section 2.4.1.1). From the results presented in figure 48 it is obvious that the anti-uPAR antibody (Figure 44, columns 3 and 4) lowered MMP9 expression significantly below control levels (Figure 44, columns 1 and 2).

![Figure 44](image-url)
The large increase in MMP9 expression when uPAR functioning was inhibited appears to further confirm and extend the results of Menshikov et al (Menshikov 2004), suggesting not only that the expression of MMP9 is highly regulated by the plasminogen/plasmin system, but also that MMP9 expression in monocytes was seen to be intimately connected with uPAR activity, and most MMP9 expression was lost in the presence of a uPAR function-inhibiting antibody expression (Rao 1995). It is interesting to note that the Ang-2/PDGF-BB-dependent upregulation of MMP9 was lost when uPAR activity was inhibited. These data strongly suggests a link between the Ang-2/PDGF-BB-dependent upregulation of uPAR, the Ang-2-dependent increased association of uPAR with integrin β2, and the observed Ang-2/PDGF-BB-dependent upregulation of MMP9.

3.6.4 MMP9 Expression and Integrin β2 Inhibition

In order to investigate the role of integrin β2 in MMP9 expression, monocytes were incubated with a function-inhibiting integrin β2 antibody with or without Ang-2 and PDGF-BB. After 15 hours the conditioned medium was analysed for MMP9 expression by gelatin zymography. The inhibition of integrin β2 removed the Ang-2/PDGF-BB-dependent upregulation of MMP9 expression (as reported in 3.3.3.2), and brought the level of MMP9 expression down to the control levels (Figure 45, columns 1 and 4). Interestingly, the addition of the function-inhibiting antibody to untreated cells did not affect the level of MMP9 expression (Figure 45 columns 1 and 3).
These results, together with those reported in 3.6.2 and 3.6.3, strongly suggested that the upregulation of MMP9 by Ang-2 and PDGF-BB was dependent on integrin β₃, and influenced by its with αPAR.

3.6.5 MT1-MMP Expression in Response to Integrin β₃ Inhibition

In order to determine the involvement of integrin β₃ in MT1-MMP expression, monocytes were incubated with a function-inhibiting integrin β₃ antibody. When the cell lysates obtained from these cells were analysed by MT1-MMP immunoblotting, it was observed that the upregulation in MT1-MMP expression in response to Ang-2 and PDGF-BB (Figure 46, column 2, as also reported in 3.3.4) was significantly decreased in samples which had been treated with the antibody (Figure 46, column 4), and that the level of MT1-MMP expression in these samples was similar to that of the control samples (Figure 46, columns 1 and 3).
These data suggested that integrin β3 is involved in the upregulation of MT1-MMP expression by human monocytes in response to Ang-2 and PDGF-BB.

3.6.6 Analysis of Possible Signaling Pathways for Ang-2/PDGF-BB Action

It was hoped that a preliminary study of the intracellular pathways involved in Ang-2/PDGF-BB-dependent upregulation of fibrinolysis could be conducted using inhibitors known to inhibit previously identified integrin-upregulated pathways, such as MAPK, PI-3-K. Inhibitors were made to further understand the signaling pathways through which PDGF-BB and Ang-2 were causing the observed upregulation in fibrinolysis in monocytes. A number of inhibitors were employed in conjunction with the serum-free fibrin invasion assay to assess whether the inhibition of various candidate pathways would be reflected in the inhibition of fibrin invasion.

3.6.6.1 Phosphoinositide 3-Kinase (PI-3-Kinase)

PI-3-kinase is a lipid kinase that phosphorylates phosphoinositides at the 3' position of the inositol ring (Dib 2000). PI-3-kinase appears to be involved in numerous aspects of leukocyte activity, including β3 integrin signaling in polymorphonuclear neutrophils (Lodgren 1993; Dib 2000). The engagement of β3 integrins was seen to increase the activity of PI-3-kinase, and
clustering of β integrins induced phosphorylation and activation of protein kinase B/Akt, a downstream target of PI-3-kinase (Lefgren 1993; Axelsson 2000; Dib 2000). It has also been shown that tyrosine-phosphorylated receptors, such as PDGF receptors, can bind to p85 and increase the catalytic activity of PI-3-kinase (Vanhaesebroeck 1997). Wortmannin is a known inhibitor of the PI-3-kinase (Prager 2004), and has successfully been used in a number of different in vitro studies.

When wortmannin was added to the fibrin invasion assay (2.3.1.1), no change in fibrin invasion was seen between Ang-2-treated samples with or without wortmannin. These results suggest that the PI-3-kinase pathway is not involved in the increase in monocyte fibrin invasion and fibrinolysis that occurs in response to treatment with Ang-2 and 1PDGF-BB. These results correspond with previous investigations which suggested that β integrins do not signal through PI-3-K (Royes-Reyes 2002). These experiments, however, were limited by the absence of a positive control, suggesting that further investigations would be necessary in order to determine the role of PI-3-K in Ang-2/PDGF-BB signaling conclusively.

3.6.6.2 Mitogen-Activated Protein Kinase (MAPK) and p53

Ang-2 adhesion to integrins in HUVEC and fibroblasts via αβ1 activates FAK and Ras/MAPK (Carlson 2001). The MAPK/ERK (extracellular signal-regulated kinase) pathway is a signal transduction pathway that couples intracellular responses to the binding of growth factors to cell surface receptors. It plays an important role in a variety of pathophysiological responses, and has been suggested to be involved in many processes considered critical to the inflammatory response and tissue remodeling (Grivuold 1995; Underwood 2000). SB239063 has previously been shown to be a potent inhibitor of p38 MAP kinase. p38 mitogen-activated protein kinases are a class of mitogen-activated protein kinases which are responsive to stress stimuli, such as cytokines, UV irradiation, heat shock and osmotic shock, and are involved in cell differentiation and apoptosis. SB239063 has been successfully used in vitro to inhibit the MAP kinase pathway in monocytes at concentrations up to 100 μM (Underwood 2000). As SB239063 has an ID50 of 44 nM, it was decided that for these inhibition experiments, it would be used at 100 nM.

When SB239063 was added to the fibrin invasion assay (2.2.1) it was observed that most of the monocyte fibrin invasion was abolished, in both the Ang-2/PDGF-BB-containing wells and the control wells. While this suggested that the MAP kinase pathway is very important
to monocyte fibrin invasion, it was not possible to determine whether the pathway was in any way involved in the Ang-2/PDGF-BB-dependent upregulation of fibrinolysis.

Apigenin, a potent inhibitor of the p33 pathway (Huang 1996) was also added to the fibrin invasion assays. Similarly to the results obtained from SB259063 experiments, when apigenin (Sigma, Steinheim, A8145) was added to the assay the invasion both the untreated cells, as well as those treated with Ang-2 and PDGF BB was severely curtailed.

Based on these observations, it was apparent that the pathways chosen for investigation were vital for cellular functioning in areas other than Ang-2/PDGF-BB stimulation. Therefore, in order to elucidate the level of involvement of the elements of each pathway, methodologies unavailable for this research would need to be employed. It is possible that the use of siRNAs to target specific elements of the pathway might provide valuable data. Furthermore, using array technology, it might be possible to investigate the upregulation of various elements of the pathways in response to Ang-2 and PDGF-BB treatment.

3.6.7 Summary

In this section, the involvement of integrin β2 in the Ang-2/PDGF-BB-mediated upregulation of monocyte fibrinolysis was further investigated. Firstly, through immunoprecipitation it was observed that, in agreement with previous reports (Simon 1996), integrin β2 was associated with uPAR under experimental conditions. Furthermore, in keeping with the observation that Ang-2 and PDGF-BB upregulated uPAR expression (3.5.3.2), cell lysates containing Ang-2 showed higher amounts of uPAR associated with the integrin.

Reports in the literature have shown that MMP9 expression in monocytes/macrophages is dependent on uPAE (Rao 1995). When uPAR expression was inhibited by function-inhibiting antibody, it was observed that the Ang-2/PDGF-BB-dependent upregulation of MMP9 (3.4.2) was lost. This strongly suggested that uPAR is involved in the Ang-2/PDGF-BB-dependent upregulation of MMP9. Furthermore, Ang-2/PDGF-BB-dependent upregulation of MMP9 was removed by the inhibition of integrin β2.

Inhibition of integrin β2 also removed the Ang-2/PDGF-BB-dependent upregulation of MT1-MMP, further confirming its role in as the Ang-2 receptor for the Ang-2/PDGF-BB-mediated upregulation of monocyte fibrinolysis.
Interestingly, when the Ang-2-integrin β immunoprecipitation was analysed by PDGFR-β immunoblotting, no PDGFR-β expression was detected in any of the samples (data not shown). This suggested that, despite reports in the literature of PDGFR-β associating with integrins on the cell surface [Niyomoto 1996; Sundberg 1996], under experimental conditions there was no physical interaction between PDGFR-β and integrin β3.

Attempts were made to identify the intracellular pathway through which Ang-2 and PDGF-BB signaled. Inhibition of the PI-3-K pathway with wortmannin did not affect monocyte invasion into fibrin with or without Ang-2 and PDGF-BB. The inhibition of the MAPK pathway by SB239063 was inconclusive, however, as monocyte fibrin invasion in both Ang-2/PDGF-BB-treated and untreated cells was abolished. While this demonstrated that the MAPK pathway is integral to normal monocyte fibrin invasion, no conclusions as to its involvement in Ang-2/PDGF-BB signaling could be elucidated. In order to gain clarity into this, it would be necessary to employ methodologies other than those available for this study.

3.7 Extension of Findings In Vitro

3.7.1 Introduction

The results reported in the previous sections strongly suggest the involvement of Ang-2 and PDGF-BB in monocyte fibrinolysis, however, the experimental designs are unable to answer some of the outstanding questions surrounding this phenomenon. Firstly, although the individual regulation of specific elements of the fibrinolytic systems suggest that the increase in fibrinolysis is a specific occurrence, it was necessary to further confirm that the increase in fibrinolysis was not an element of a global increase in proteolysis. In order to do this, the fibrin invasion experiments were repeated using Growth Factor-Reduced Matrigel (GFR Matrigel) as an alternative protein substrate. As GFR Matrigel does not contain fibrinogen, but is composed of a number of other ECM proteins, increased invasion by Ang-2/PDGF-BB-treated monocytes would suggest a more global upregulation of proteolysis, rather than a specific increase in fibrinolysis.

Many of the integrin family functions are redundant between individual members, and a number of different integrins can bind the same ligand [Hynes 1992; Rüegg 2001]. Therefore, although β3 integrin expression is restricted to leukocytes [Dib 2000], it is possible that Ang-2 may signal through one of the other integrins on non-leukocytic cells. In order to investigate this possibility, the effect of Ang-2 and PDGF-BB on fibrinolysis was investigated
in three PDGFR-β-expressing cell types involved in wound healing – endothelial cells, smooth muscle cells and fibroblasts.

Although unlikely, it was possible that the effects of Ang-2 and PDGF-BB on human monocytes described above could be a species-specific phenomenon. Thus, in order to determine whether the results described above were species specific, the serum-free fibrin invasion assay (2.2.7) was repeated using monocytes isolated from male Wistar rats and baboons.

3.7.2 In Vitro Analysis of Ang-2- and PDGF-BB-Mediated GFR Matrigel Lysis

Matrigel is a commercially-available soluble basement membrane extract from the Engelbreth-Holm-Swarm tumour that gels at room temperature to form a genuine reconstituted basement membrane. The major components of Matrigel matrix are laminin, collagen IV, entactin and heparan sulfate proteoglycan (Table 15B). Matrigel is commonly used in vitro as a substrate for migration assays, as it is a homogenous and compact medium with a composition similar to the basement membrane of endothelial cells. In addition, it has also already been used as a support for in vivo angiogenesis assays (Anghelina 2002). Growth factor Reduced (GFR) Matrigel, as used in the following experiment, differs very little from Matrigel (Table 15B) in composition, but is growth factor depleted (Table 15A).

![Table 15](image)

**Table 15.** Detailing various aspects of GFR Matrigel. A. The growth factors present in Matrigel and GFR Matrigel. B. ECM components of Matrigel and GFR Matrigel.
Matrigel has previously been used in a variety of studies involving monocytes (Whitte 2001), although studies using GFR Matrigel are less common (as the product is relatively new). GFR Matrigel was chosen as a suitable non-fibrin substrate in an effort to test whether the upregulation of fibrin degradation caused by Ang-2 and PDGF-BB was also seen in other forms of proteolysis. Therefore, the serum-free invasion assay (2.3.2.1) was repeated; however, the fibrin clot was replaced with a GFR Matrigel clot of similar dimensions. Cells were seeded on top as before, and the experiment incubated at 37°C.

Three donors, all in triplicate, had minimal cells in the bottom compartment of all treatments after two days of incubation, and no significant difference between the treatments after four days of incubation (data not shown). The incubation times necessary for cells to penetrate the GFR Matrigel was notably longer than for fibrin (4 days versus 19 hours), however, judging by references in the literature, this is not unexpected (Shawley 1980 - 5 days for an 8μm layer; Zhu 2005 - 24 hours for a “thin layer”). As shown in table 15A above, GFR Matrigel does not contain plasminogen. The absence of an extracellular supply of plasminogen could account for the slow invasion of monocytes into the GFR Matrigel clots in vitro.

These observations appear to suggest that the effect seen during monocyte exposure to Ang-2 and PDGF-BB is probably limited to fibrinolysis, and is not likely to be part of a general proteolytic upregulation. As the untreated monocytes, however, penetrated the Matrigel very inefficiently, it is not possible to draw any conclusions from these experiments. Further experiments using individual ECM proteins would be necessary to determine the nature of this observation.

3.7.3 In Vitro Analysis of the Effect of Ang-2 and PDGF-BB on Fibrinolysis in Other Cell Types

In order to investigate whether Ang-2 and PDGF-BB affected the fibrinolytic mechanisms of other cell types involved in wound healing, the fibrin invasion assay described in 2.3.1.1 was repeated using ECs, fibroblasts, and smooth muscle cells (SMCs) (obtained as described in 2.6.2). As mentioned above, it was thought possible that in the absence of β1 integrins, Ang-2 might signal through one of the other integrins. ECs, SMC and fibroblasts have all previously been reported to express PDGFRβ (Crosby 1990; Tada 2003; Kyan 2005). Furthermore, while ECs are known to express Tie-2 (Maisonpierre 1997), they have also been shown to adhere to Ang-2 in Tie-2-unrelated adhesion (Carlson 2001). Therefore, was
thought possible that the Ang-2/PDGF-BB increase in fibrinolysis seen in monocytes might also be present in other cell types.

Figure 47. Detaching from invasion in smooth muscle cells (SMC), human endothelial cells (EC) and fibroblasts after 3 and 4 days of incubation with Ang-2 in a serum-containing environment. Neither SMC (pair 1 and 2) nor fibroblasts (pair 3 and 4) displayed a significant difference between fibrin invasion in the presence or absence of Ang-2. ECs did not show any fibrin invasion after 4 days (pair 3 and 4): p > 0.05. n = 3 experiments in triplicate.

All three different cell types took considerably longer to invade the fibrin clot under serum-containing conditions in comparison to the results obtained with monocytes above (3.2.2.1). The Transwells containing the fibrin clot and the seeded cells were incubated for either three or four days before any fibrin invasion was observed. Due to the larger sizes of these three respective cell types, fibrin invasion was quantified by counting the cells appearing on the underside of the semi-permeable membrane (3.2.3), and not the cells in the bottom compartment. The average number of cells per field (under 10x magnification) is presented on the figure above (Figure 47).

After four days of incubation, neither the endothelial cells (both HSEEC and MVFC) in the control wells nor those treated with Ang-2 were seen to invade the fibrin clot. Upon examination of the fibrin, very few zones of lysis were seen, suggesting very low levels of fibrinolytic activity. After three days, fibroblasts had not invaded the fibrin clot, and no cells were present on the membrane. However, on the fourth day of incubation cells were observed to have migrated through the clot, although the addition of Ang-2 did not seem to make a significant contribution to the speed of migration and thus the fibrinolytic activity.
SMCs displayed considerable proteolytic activity throughout the experiment, with zones of lesions visible in the fibrin clot from the second day of incubation. Cells were present on the membranes on both days three and four, however the addition of Ang-2 to the culture medium did not seem to significantly increase the number of cells.

These results suggest that the increase in fibrin invasion seen in response to Ang-2 under in vivo conditions approximates the in vivo environment, thus implying that all cell types equally. EPC and fibroblasts expressed Ang-2, Ang-3, and GM-CSF under experimental conditions.

It is possible, therefore, that the Ang-2-dependent fibrin invasion is unique to monocytes, or is characteristic of cells expressing the proliferation, and further investigation involving other Ang-2-expressing leukocytes would be necessary.

3.7.4 In Vitro Analysis of the Effect of Ang-2 and PDGF-BB on Animal Cells

Before testing the effect of Ang-2 and PDGF-BB on monocytes in vitro, it was ascertained that the invasion assay should be repeated using normal cells. Positive results from animal invasion assays should be repeated using normal cells. Positive results from animal cells would suggest that the Ang-2 effect observed were not species specific, or indeed a unique feature of the population group sampled. Monocytes from two different species were analyzed. Rat monocytes were selected as a member of the primates family. All animal work was approved by both the Department of Surgery Research Committee and the Animal Ethics Committee of UCT.

3.7.4.1 Analysis Using Rat Peripheral Blood Monocytes

Because of the low volumes of blood obtainable from a rat, many researchers isolate monocytes from the spleen of the animal (personal communication Dr. Marion Schuler. Dept of Surgery, UCT, RSA). In these experiments, however, the monocytes were isolated as described in 2.1.1 from whole blood. This blood was obtained through exanguinations by carotid puncture (25-30 ml) and the blood from three male Wistar rats (28 months old) were pooled for each isolation.
When the fibrin invasion assay (2.2.7) was repeated using rat monocytes, a significant increase in fibrin invasion was seen in the presence of (human) Ang-2 and PDGF-BB (475% increase, p <0.05), which was not present in any of the other treatments (Figure 18). The increase in fibrin invasion in the presence of these two growth factors was considerably larger than that observed when the experiment was conducted using human monocytes.

3.7.4.2 Analysis Using Baboon Peripheral Blood Monocytes

Baboon blood was generously provided by the Cardio-Thoracic surgery department (UCT, South Africa) from baboons, which had been obtained by the department for an ethically approved study of cardio-implants. Aside from Vitamin B and Ivomec, the baboons were otherwise medication-free at the time of blood drawing. Blood was drawn from the forearm of the animals, and monocytes isolated as described in 2.1.1.
As demonstrated in the figure above (Figure 49), Ang-2 and PDGF-BB significantly increased the level of baboon monocyte fibrin invasion in vitro. The increase in invasion seen in these experiments was far above that seen in other species (940% versus 475% for rats and 200% for humans).

### 3.7.5 Summary

Investigations using GFR Matrigel instead of fibrin showed very little monocyte invasion in the presence of Ang-2 and PDGF-BB. These results suggested that Ang-2 and PDGF-BB specifically upregulate fibrinolysis in monocytes, and do not affect other proteolytic mechanisms, however further investigations would be necessary to confirm the observation.

Furthermore, by comparing fibrin invasion between monocytes and other wound-related cell types, such as endothelial cells, smooth muscle cells and fibroblasts, suggested that either the upregulation of fibrinolysis in the presence of Ang-2 and PDGF-BB seen in monocytes did not translate to other cell types, or that this upregulation was restricted to cell types expressing both PDGFR β and integrin β₈.

When the effects of Ang-2 and PDGF-BB on monocytes were investigated in other species, once again it was noted that Ang-2 and PDGF-BB in combination caused a significant upregulation in fibrin invasion, while not having an effect individually. These data strongly
suggested that the combined effect of Ang-2 and PDGF-BB on monocyte fibrinolysis was not a species-specific phenomenon. Furthermore, it was noted that the increase in fibrin invasion varied between the species (200% in humans, 465% in rats, and 940% in baboons). While no immediate explanation can be offered for this observation, it is possible that the effects of the growth factors differ slightly between species.

3.8 In Vivo Analysis of Model

3.8.1 Introduction

Most in vitro assays have very reductionist readouts, and, while powerful, are limited for use in the study of complex cellular interaction phenomena. Most in vivo assays, however, are either not quantitative or they are restricted to endpoint readouts that do not allow an appreciation of the dynamic 3-dimensional spatiotemporal order of angiogenic processes (Augustin 2001). Because of these limitations, it is sometimes difficult to translate in vitro data into in vivo results.

The in vivo model utilized to test the in vitro Ang-2/PDGF-BB-dependent increase in monocyte invasion into fibrin reported above was adapted from one previously used successfully by colleagues in the lab. In his PhD thesis (Dr Christian Schmidt, CVRU, UCT, RSA, 2007), Dr Schmidt investigated the immobilization of growth factors on a heparin-coated polyurethane (hPU) disc as a novel method for growth factor delivery in vivo. Due to the reversible nature of the growth factor-heparin binding, the growth factors gradually diffused into the surrounding area, causing cellular infiltration into the disc. After a predetermined amount of time, the disc was explanted, and the level of cellular inlax into the disc determined using immunocytochemistry. In the experiments reported below, both Ang-2 and PDGF-BB were immobilized on the hPU discs following protocols developed by Dr Christian Schmidt and Dr Neil Davies (CVRU, UCT, RSA). These discs were then encapsulated in a fibrin gel and implanted subcutaneously in male Wistar rats.

The hPU discs were also previously investigated as a carrier for recombinant adeno-associated viruses (rAAV) by Dr Christian Schmidt. In these experiments the rAAV was loaded into the hPU discs inside a matrix, such as fibrin, polyethylene glycol (PEG), and collagen. It was found that inside these matrices, the rAAV could be retained inside the disc in vitro for up to 48 hours. It was also seen that in the presence of serum, the rAAV retained effective infectivity for up to 21 days. The success of this model in vitro suggested that it
could be used in vivo as an alternative method of growth factor delivery with the transduced infiltrating cells providing an alternative source of recombinant growth factors to the direct loading of growth factor proteins into the heparin-coated discs. Due to the considerable quantities of growth factor proteins needed for in vivo analyses using the hPU model, using rAAVs was investigated as a viable alternative for the delivery of Ang-2 and PDGF-BB in vivo.

3.8.2 In Vitro Analysis of Heparin-Coated Polyurethane Discs

Previous research conducted by Dr Neil Davies and Dr Christian Schmidt (CVRU, UCT, RSA) showed that PDGF-BB bound with high affinity to the heparin, and 95% of recombinant PDGF-BB added to a heparin disc remained bound for after a week of incubation in PBS. As no previous data existed on the binding affinity of Ang-2 to heparin, this was investigated in vitro as described above (2.7.2). Ang-2 was suspended in PBS at a concentration of 1 μg/ml and incubated with the hPU discs overnight at 4°C. The discs were then washed 3 times with PBS and incubated for another 48 hours in PBS at 4°C. The Ang-2 remaining in the disc after the incubation was eluted in 10 mM HCl for half an hour at room temperature. These samples were then freeze-dried and analysed by silver staining (2.3.4). As shown below (Figure 50), after vigorous washing and incubation, detectable amounts of Ang-2 (about 100 μg) remained in the disc after 48 hours.

![Figure 50: Ang-2 remaining in hPU disc after washing and incubation. Lane 1 represents gel containing Ang-2, Lane 2 is blank, and lane 3 contains elution from hPU disc.]

3.8.3 Sub-Cutaneous Implantation of Growth Factor-Carrying Heparin Discs

The hPU discs were prepared with Ang-2 and PDGF-BB as described in section 2.7.1. The growth factor-containing hPU discs were then coated with 4 mg/ml fibrin (2.7.3). The fibrin coating of the hPU discs was analysed through scanning electron microscopy (with the kind help of Ms Nazli Samadien, CVRU, UCT), and high-resolution photography (with the kind help of Mr Johann Egelhard, CVRU, UCT). Although the fibrin was seen to set very well around the discs (Figure 51), manipulating the discs proved to be a serious flaw in the experimental design, and a number of fibrin layers were damaged during the subcutaneous implantation into the rats.
Figure 51. Fibrin-coated hPU discs. As can be seen in both A (15x) and B (35x), the fibrin coating the disc formed an even, regular layer against the surface of the hPU disc. C and D are top and side views of the disc coated with fibrin and stained with Brilliant Blue. Pictures taken with high-resolution photography.

When the discs were explanted after 4 days (as described 2.7.5), the size of the fibrin layers remaining was highly variable both between and within disc treatments. When the discs
were sectioned and analysed with haematoxylin and eosin. Cellular infiltrates detected in the fibrin layers. These cellular infiltrates were further analysed by ED1 immunohistochemistry (2,7,6) in order to identify the percentage monocyte/macrophages in the infiltrates. The extreme variability between the sizes of the fibrin capsules of the samples, however, made any conclusions impossible, and it was concluded that without development beyond the scope of this thesis, this in vivo model was not informative.

3.8.4 Development of Recombinant Adeno-Associated Viruses

As mentioned in 3.7.7, rAAV viruses were developed in order to provide an alternative to direct growth factor loading into the hPUs discs. A rAAV carrying the gene for green fluorescent protein (GFP) was developed as a positive control for level of infectivity both in vitro and in vivo. As shown below (Figure 52), both Ang-2-carrying, and GFP-carrying rAAVs were successfully manufactured and shown to be infective in vitro, as determined by Ang-2 immunoblotting (Figure 52A) and GFP fluorescent microscopy (Figure 52B). Unfortunately, studies conducted by Dr Christian Schmidt showed that while the viruses could be adequately immobilized on the hPUs discs in vitro, these results could not be reproduced in vivo, and very little infectivity could be detected in the tissue surrounding the discs, as determined by GFP expression.

![Figure 52. Recombinant AAV display detectable transduction in vitro. A. rAAV-Ang-2 causes transduced CHO cells to produce Ang-2 in vivo. Lane 1 is a sample of conditioned medium from transduced CHO cells. Lane 2 is a 500ng standard of Ang-2. Lane 3 is a sample of conditioned medium from control CHO cells. B. rAAV-GFP causes transduced CHO to produce GFP in vivo. Cells expressing GFP (arrow) were detected using fluorescent microscopy.](image-url)
3.8.5 Discussion of Failure of In Vivo Models

A limitation which precluded many other in vitro experimental models was the need to quantify macroocyte infiltration at a very early stage of wound healing. One of the advantages of using the hPU discs was that the subcutaneous positioning of the discs in the rate made for very convenient implantation and explantation.

One of the major shortcomings of the fibrin-coated hPU disc model was the difficulty experienced by the surgeon during implantation and explantation of the discs, due to the softness of the fibrin gels. While increasing the concentration of the fibrin gels would creating a harder and more durable gel, literature exists to suggest that the concentration of fibrin potentially affects the rate of macroocyte migration into the gels (Ciano 1986). It is likely too that the fragility of the fibrin also contributed to the extreme variability seen in the fibrin layers after the implantation.

The possibility of transfecting macrocytes with siRNA in order to inhibit PDGFR-β and/or β2 integrins (as the receptors for PDGF-BB and Ang-2) was also investigated, and some references in the literature report successful transfections of macrocytes using the DOTAP liposomal transfection reagent available from Roche (Roche, 11811177001) (Kroener-West 1999). Many other reports (as well as personal communications with various other researchers and reps), however, suggest that transfecting leukocytes is a process of considerable difficulty, due to their innate ability to degrade foreign DNA and RNA. This led to the decision that without extensive in vitro characterization, any in vivo investigations carried out using transfection methodology would produce potentially conflicting results, and was therefore not suitable for use in these experiments.
Further Discussion and Conclusions

The work of this thesis explores the cooperative stimulation of Ang-2 and PDGF-BB on peripheral blood monocytes. When administered together in vitro, Ang-2 and PDGF-BB together stimulate an upregulation in fibrin invasion and fibrinolysis, which was absent when either growth factor is applied singly. Upon investigation, it was observed that the increase in invasive phenotype was due to the upregulation of elements of both the plasminogen/plasmin and the MMP systems.

While both Ang-2 and PDGF-BB have been reported to act cooperatively with other growth factors (Hanahan 1997; Maisonpierre 1997; Etoh 2001; Lobov 2002; Russo 2002; Fiedler 2006), the interaction of these two is a novel observation. Ang-2 is released from the Weibel-Palade bodies of endothelial cells in response to a number of different factors (Fiedler 2004; Fiedler 2006), such as thrombin (Huang 2002), while one of the sources of PDGF-BB is known to be the α-granules of platelets, and is released during platelet degranulation (Heldin 1999). These two release mechanisms suggest a clear conjunction in the delivery of these two growth factors during fibrin clot formation, and the intiation of repair. This congruence of events also occurs during pathological remodeling situations, such as myocardial infarction (Frangogiannis 2006).

Increased fibrin invasion by monocytes in response to Ang-2 and PDGF-BB could be inhibited by protease inhibitors of both major fibrinolytic systems. While monocytes have been previously reported to express many elements of these systems (as discussed in 1.3.2) (Loscalzo 1996; Bar-Or 2003; Pierleoni 2003), these studies show that in response to Ang-2 and PDGF-BB, two membrane-bound proteins involved in pericellular fibrinolysis (Ellis 1997; Hiraoka 1998), uPAR and MT1-MMP, were upregulated. The inhibition studies conducted for both uPA and its receptor uPAR further suggested their involvement in the Ang-2/PDGF-BB-dependent fibrin invasion. Upon examination it was noted that monocytes migrated through the fibrin prior to completing lysis of the clot, further suggesting that pericellular lysis was central to the increased fibrinolysis. Indeed, tPA, MMP3 and MMP7 - fibrinolysins previously reported to be secreted by monocytes (Lijnen 2002) - were not
expressed significantly in the cells under experimental conditions, further suggesting a pericellular fibrinolytic response.

Both uPAR and MT1-MMP have previously been shown to be involved in the recruitment of monocytes to sites of inflammation (Estreicher 1990; Matias-Roman 2005). It is intriguing to note that MT1-MMP was found to be necessary for proper extravasation of monocytes through TNF-α-activated endothelium. This suggests that Ang-2 plays a central role in directing the recruitment of monocytes to a site of vascular disturbance through not only regulating adhesion to the endothelium (Fiedler 2006), but also enabling the cell to move through the endothelial layer and invade the underlying matrix. Indeed, assessing the effects of Ang-2 and PDGF-BB on monocyte extravasation would potentially be an interesting avenue of investigation for future research.

Although the role of MMP9 in fibrinolytic activity is not as established as those of the above proteins, recent studies have shown MMP9 to degrade fibrin in vitro (Hiraoka 1998), while increased fibrin deposits have been observed in MMP9-null mice (Lelonght 2001; Lambert 2003). MMP9 has also been shown to affect leukocyte trafficking during inflammation (McQuibban 2000; Wetzler 2000; Menshikov 2004; Greenlee 2006), and is known to be important for leukocyte invasion, during which it is associated with integrin β3 (Stefanidakis 2006). It is also important to note that MMP9 has recently been proposed as an element of the angiogenic switch (Bergers 2000; Nozawa 2006), being highly influential in the release of growth factors from ECM sequestration. Therefore, the upregulation of MMP9 expression indicates increased induction of vessel ingrowth during wound healing.

While Tie-2 expression has previously been reported on cells of hematopoietic lineage, such as neutrophils and endothelial precursor cells (Ikeda 2004; De Palma 2005; Lemieux 2005; Modarai 2005), no Tie-2 expression was found in monocytes under any of the conditions studied in these experiments. Recent reports have suggested that Tie-2 is expressed by a subset of monocytes (Venneri 2007), however, no data was found to support this observation. It is possible that differing isolation and culture techniques may explain these divergent results.

Ang-2 has previously been found to interact with a number of cell types via mechanisms other than Tie-2 binding. Indeed, the highly conserved COOH-terminal fibrinogen-like domain of the angiopoietins implies a functional association with the integrin receptor family (Yokoyama 2000; Camenisch 2002; Hu 2006). Fibroblasts, as well as skeletal myocytes, were
found to bind to surfaces coated with Ang-2 (Carlson 2001; Dallabrida 2005), and this adhesion was mediated by integrins αv, α6, β1, and β3. Alternative evidence from cancer studies shows that Ang-2 is able to induce the expression of MMP2, MT1-MMP and laminin-V in Tie-2-deficient glioma cells, of which MMP2 was seen to be mediated by Ang-2 via interaction with integrin αβ1 through the focal adhesion kinase pathway (Hu 2006). Ang-2 binding to monocytes was investigated by immunoprecipitation, and Ang-2 was found to be associated to integrin β3, a leukocyte-specific integrin (Dib 2000; Grisar 2001). When an integrin β3 function-inhibiting antibody was added to the fibrin invasion assay, it was seen that the inhibition of integrin β3 function removed the Ang-2/PDGF-BB-dependent upregulation of fibrin invasion. This strongly suggested that the immunoprecipitation results reflected a functional association between Ang-2 and integrin β3. Although integrin β3 has been previously identified as being involved in a number of different fibrinolytic mechanisms (as discussed in 1.3.2), when the function-inhibiting antibody was added to the control cells, no effect was observed. This suggested that this interaction was specific to integrin β3 and Ang-2. Furthermore, the Ang-2/PDGF-BB-dependent upregulation of MT1-MMP and MMP9 was seen to be lost during integrin β3 inhibition. Further research is needed, however, to identify the α-integrin to which integrin β3 is associated under these circumstances, and therefore to further link this research in with the body of literature on integrins and monocyte/macrophages.

Further analysis of the integrin β3 immunoprecipitates revealed that uPAR was also associated to the integrin. While the formation of a uPAR/integrin β3 functional unit had previously been described (Simon 1996; Sitrin 1996), it was observed that in the presence of Ang-2, increased amounts of uPAR were associated with integrin β3. This, in conjunction with the Ang-2/PDGF-BB-dependent upregulation of uPAR expression also reported in this study, suggests that Ang-2 binding to integrin β3 in the presence of PDGF-BB not only causes an upregulation of uPAR, but also that the upregulated uPAR is physically associated with the Ang-2-binding integrin. The removal of Ang-2/PDGF-BB-dependent upregulation of MMP9 expression by inhibition of uPAR function further suggested that uPAR is integral to the Ang-2/PDGF-BB signaling.

Monocytes have previously been reported to respond to PDGF-BB, and a number of studies have reported the expression of PDGFR-β (Krettek 2001). Both RT-PCR and PDGFR-β ELISA analyses of PDGFR-β expression showed an increase in receptor expression during Ang-2 treatment both with and without PDGF-BB. These observations strongly suggest that
one of the ways in which Ang-2 and PDGF-BB interact to upregulate monocyte fibrinolysis is through the Ang-2-dependent initiation of PDGFR-β expression on the cell surface.

Both PDGF-BB and PDGFR-β have been reported to interact with integrins. PDGFR-β associates with activated insulin and integrin αβ₁, thus potentiating the biological activity of PDGF in fibroblasts (Schneller 1997). PDGF-BB, on the other hand, has been found to influence the clustering of integrin β₁ in SMC (Ahlen 2004), as well as inducing MMP9 production in SMC simultaneously exposed to an integrin αβ₃ ligand (Bendeck 2000). However when the integrin β₂ immunoprecipitate was analysed by PDGFR-β immunoblotting, no association was found between the two receptors.

Figure 53 is a schematic diagram of the proposed interactions between the elements studied in this thesis, based on experimental results. The invasion assays showed that Ang-2 and PDGF-BB cooperatively stimulated monocyte fibrinolysis, while the inhibitor-containing invasion assays suggested that elements of both the plasminogen/plasmin (uPAR and uPA) and MMP fibrinolytic systems were involved. The involvement of both uPAR and uPA in the observed upregulation of monocyte fibrinolysis also suggested an increase in the conversion of plasminogen to plasmin.

Immunoprecipitation and function inhibition suggested a physical and functional link between Ang-2, integrin β₂. Further immunoprecipitation of integrin β₂ with uPAR, as well as the Ang-2-dependent upregulation of this association extended the previously reported association of integrin β₂ with uPAR (Simon 1996) to include the observed Ang-2/PDGFB-BB-dependent upregulation of uPAR seen by immunoblotting. Previous investigations (Menshikov 2002; Menshikov 2004) have shown that MMP9 expression in monocytes is dependent on uPAR, and indeed, in this study, this was shown to occur under these experimental conditions. It therefore seemed probable that Ang-2 binding to integrin β₂, via uPAR was responsible for the observed upregulation of MMP9. Integrin β₂ binding to Ang-2 was also shown to upregulate the expression of PDGFR-β, suggesting one of the ways in which Ang-2 and PDGF-BB act cooperatively in monocytes. Ang-2 and PDGF-BB together were shown to upregulate the expression of MT1-MMP, although the manner in which this occurred was not investigated.
Previous investigations have reported the activation of both the MAPKp42/44 and the AKT pathways through the interaction of Ang-2 with integrins (Carlson 2001; Dallabrida 2005). Furthermore, in fibroblasts it has been noted that PDGF-BB produced a marked, transient activation of the ERK class of MAPK when integrins were both aggregated and occupied by a ligand (Miyamoto 1996). A brief investigation into signaling pathways did not provide conclusive data, due to inappropriate methodologies. The inhibition of the PI-3-K pathway did not affect Ang-2/PDGF-BB-dependent upregulation of fibrin invasion, suggesting that this intracellular pathway was not involved in the reported phenomenon, although further investigations would need to be conducted before this could be established conclusively.

As all the proteolytic elements described above have been previously implicated as markers of activation of monocytes to macrophages (Pierleoni 2003), it would be necessary to determine whether Ang-2 and PDGF-BB increased specific elements of the fibrinolytic pathways. Interestingly, when monocyte invasion into Matrigel in response to Ang-2 and PDGF-BB was quantified it was noted that the growth factors did not enhance the invasion of cells into ECM substrates. Unfortunately, as monocyte invasion into Matrigel was not pronounced in the control samples, it was not possible to draw conclusions from these experiments, although they did seem to suggest that Ang-2 and PDGF-BB did not mediate a broad-spectrum invasive profile, and did not seem to point towards heightened activation. Further experiments would need to be conducted using a variety of purified ECM substrates, in order to pinpoint the influence of Ang-2 and PDGF-BB on monocyte proteolysis. Furthermore, as monocyte activation is characterized by increased cellular metabolism,
mobility, lysosomal enzyme activity and cytocidal capacity (Ross 2002) it might be useful to examine these aspects of activation in terms of treatment with Ang-2 and PDGF-BB.

When the fibrin invasion assays were repeated using peripheral blood monocytes from both rat and baboons, it was seen that the increase in invasion in response to Ang-2 and PDGF-BB remained similar, showing that this phenomenon is not a species-specific trait. Furthermore, despite references suggesting that the functions of integrins are in part redundant (Hynes 1992; Ruegg 2004), increased fibrinolysis in response to Ang-2 and PDGF-BB was not seen in SCMs, ECs, or fibroblasts - all of which express PDGFR-β, but not integrin β2. It remains to be investigated whether other β2-expressing leukocytes respond to Ang-2 and PDGF-BB in a similar manner to monocytes.

It is worth reiterating that Ang-2 appears to function in an interconnected manner with other growth factors (Hanahan 1997; Fiedler 2006). It has previously been shown to stimulate angiogenesis in conjunction with VEGF (Maisonpierre 1997; Lobov 2002), inflammatory cell adhesion in conjunction with TNF-α (Fiedler 2006), and now, increased monocyte proteolytic invasion through PDGF-BB. This suggests that Ang-2 is a key regulator of wound healing, enabling other necessary growth factors to elicit their required response. The specific interaction between Ang-2 and integrin β2 further strengthens the proposal that Ang-2 is a pro-inflammatory mediator of the initial steps of inflammation and pathological angiogenesis (Fiedler 2004; Lemieux 2005; Roviezzo 2005; Fiedler 2006; Imhof 2006). Indeed, mice deficient in Ang-2 could not elicit an inflammatory response to immune challenge, a response restored by the administration of recombinant Ang-2 (Fiedler 2006).

The identification of Ang-2 and PDGF-BB as factors that may be involved in the intitial recruitment of monocytes to a wound site suggests that they could prove to be useful, clinical targets in the drive towards reduction of scarring during tissue repair. Given the increasing importance of scar prevention in regenerative medicine, together with the recent realization that the presence of macrophages throughout the duration of the wound-healing response may not be required, suggests that manipulation of their recruitment may prove increasingly important for engineered tissue regeneration (Martin 2003; Duffield 2005).

Furthermore, MMP inhibitors have been actively tested in tumour therapy over the last few years, but their potential in inflammatory diseases has just started to be explored (Matias-Roman 2005). Understanding how MMPs are involved in inflammation, and what triggers their expression might assist in producing novel and alternative therapeutic approaches to a
variety of conditions. In addition, the recent observation that integrin β2-deficient mice exhibit blunted inflammatory responses (Pluskota 2003) could also produce a viable avenue of therapeutic intervention, in which the selective inhibition of integrin β2 might provide a means to controlling aspects of inflammation.

Previous in vitro Ang-2 experiments have yielded many variable results, highlighting the difficulty of drawing conclusions from any one system (Jones 2003). Nevertheless, this thesis, together with a rapidly growing body of evidence support the involvement of Ang-2 in many aspects of wound healing, and highlights the importance of understanding the roles of Ang-2 in both inflammation and angiogenesis.


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