ROLE OF COAGULATION CASCADE PEPTIDES IN THE REGULATION OF FIBROBLAST PROLIFERATION

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IT is not the critic who counts,
Not the strong man who points out where the weak man stumbled,
Or where the doer of deeds could have done better.
The credit belongs to the man in the arena,
Whose face is marred with sweat and blood,
Who tires and fails, yet tries again,
Who at best knows the triumphs of high achievements,
And who at worst, if he fails, fails whilst daring greatly,
So that his place shall never be with those cold and timid souls
Who know neither victory nor defeat.

Anon.

Cette thêse est dédiée à mon frère Benoît

ABSTRACT

Fibroblast proliferation and extracellular matrix deposition play a critical role in tissue repair and fibrosis. These functions are thought to be modulated by cytokines and growth factors, but these mechanisms are only partially understood. Tissue injury is associated with blood vessel disruption and the activation of coagulation cascade factors VII, IX, X and II (prothrombin) which result in the cleavage of blood fibrinogen into a haemostatic fibrin clot. In addition, increased levels of active coagulation cascade factors and fibrin deposition are associated with fibrotic diseases of the lung, liver, kidney, heart and vasculature.

Recently, thrombin has been shown to promote fibroblast chemotaxis, proliferation and procollagen production *in vitro* and it has been proposed that it may contribute to tissue repair and fibrosis. Little is known about the cellular effects of the other coagulation cascade factors. The aim of this thesis is to study the effects of coagulation factors VIIa, IXa, Xa and fibrinogen cleavage products on fibroblast proliferation *in vitro*.

This thesis shows that factor Xa is mitogenic for fibroblasts and the kinetics of this effect are equivalent to that of thrombin. Studies with specific inhibitors and antibodies demonstrated that this effect is dependent on its catalytic site, but independent of thrombin generation. Furthermore, this is mediated by platelet-derived growth factor production and autocrine stimulation similarly to thrombin. In contrast, factor IXa and fibrinogen-derived peptides have no effects, but factor VIIa and stimulates proliferation at high concentrations. Further studies of receptor expression, activation and intracellular calcium signalling showed that factor Xa stimulates proliferation via binding to effector cell-protease receptor-1 and the proteolytic activation of protease-activated receptor-1.

In conclusion, this thesis demonstrated for the first time that coagulation cascade factors Xa and VIIa are mitogenic for fibroblasts, but not all peptides generated during blood coagulation. Furthermore, the mitogenic effect of factor Xa is mediated by a novel dual receptor system involving binding to effector-cell protease receptor-1 and activation of protease-activated receptor-1. These observations suggest that factor Xa may play a role in the regulation of fibroblast proliferation during normal tissue repair and fibrosis.

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LIST OF ABBREVIATIONS

ARDS	Adult respiratory distress syndrome
ASN	Antistasin
BAPTA-AM	1,2-bis-(2-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid acetoxymethyl ester
bFGF	Basic fibroblast growth factor
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary DNA
DAG	Diacylglycerol
DEGR-ck	Dansyl-Glu-Gly-Arg chloromethylketone dihydrochloride
DMEM	Dulbecco's modification of Eagle's mediumAntistasin
DNA	Deoxyribonucleic acid
d.p.m.	Desintegrations per minute
ECM	Extracellular matrix
EDTA	Ethylenediaminetetracetic acid
EPR-1	Effector cell protease receptor-1
FGN	Fibrinogen
FBN	Fibrin
fXa	Coagulation factor X (active)
Gla	γ-carboxyglutamic residue
HBS	Hepes-buffered saline
HFL-1	Human foetal lung fibroblasts type 1
HPLC	High pressure liquid chromatography

hyp	Hydroxyproline
Ig	Immunoglobulin
mAb	Monoclonal antibody
MAPK	Mitogen-activated protein kinase
M_r	Apparent molecular mass
mRNA	Messenger ribonucleic acid
NCS	Neonatal calf serum
pAb	Polyclonal antibody
PAGE	Polyacrylamide gel electrophoresis
PAR-1	Protease-activated receptor-1
PBS	Phosphate-buffered saline
PDGF-AB	Platelet-derived growth factor-AB
PKC	Protein kinase C
PL	Membrane phospholipid
PPACK	Dansyl-Phe-Pro-Arg chloromethylketone dihydrochloride
RGD	Arginine-Glycine-Aspartic acid
RNA	Ribonucleic acid
RT	Room temperature
rTAP	Recombinant tick anticoagulant peptide
SDS	Sodium dodecyl sulphate
s.e.m.	Standard error of the mean
TGF-β1	Transforming growth factor-β1
tPA	Tissue-type plasminogen activator
uPA	Urokinase-type plasminogen activator
uPAR	Urokinase-type plasminogen activator receptor

CHAPTER 1

INTRODUCTION

One novel aspect of tissue healing that has emerged over the last ten years is the possibility that coagulation cascade factors may not only mediate blood coagulation, but also regulate cellular functions at sites of trauma. These peptides interact directly with cell surface receptors and stimulate secondary mediators. Since fibroblasts play a crucial role in the regulation of connective tissue metabolism in both normal responses to injury and fibrotic disorders, their role and the mechanisms that regulate their activity are of particular interest. This thesis will investigate the hypothesis that the coagulation cascade which is activated specifically at the time and site of tissue injury, may be a source of peptides which control connective tissue formation and regulate fibroblast proliferation and extracellular matrix (ECM) production.

In this introduction, the notions of tissue repair and fibrosis will be defined and the main phases of tissue repair in response to injury will be reviewed with respect to both normal healing and fibrotic disorders. In addition, the structure, role and mechanism of activation of the coagulation factors will be reviewed in detail. Specific information relevant to each chapter will be provided in separate introductions.

1.1 TISSUE REPAIR AND FIBROSIS

Tissue injury causes the disruption of the complex architecture of organs and impairs their function. To re-establish the integrity of the injured tissue, repair proceeds as a time-dependent series of events. Proliferating cells migrate towards the wound site, repopulate the area and replace non-viable material by *de novo* deposition of connective tissue.

The regenerated mesenchyme, or *scar tissue*, is the result of a fibroproliferative response during which cell involvement is tightly regulated and includes migration, replication, matrix biosynthesis and degradation (Clark, 1996, for review; Mutsaers *et al.*, 1997). Scar tissue rarely displays the same degree of functionality as the original tissue and tissue repair is not equivalent to organ regeneration. However, scarring restores the mechanical integrity of damaged organs and allows them to continue performing their vital functions.

1.1.1 THE MAIN PHASES OF TISSUE REPAIR

The progression of normal tissue repair can be broken down into the phases of haemostasis, inflammation, granulation tissue formation and remodelling. These phases can be differentiated according to the involvement of particular groups of cells and the quality of the restored tissue but they also overlap. The main inflammatory cell types will be reviewed as well as the particular roles of growth factors and cytokines during tissue repair.

Haemostasis

Tissue damage is generally associated with injury to the underlying vascular system and leads either directly or indirectly to the extravasation of blood cells and proteins. An important initiating step of tissue repair is the formation of a haemostatic blood clot, composed mainly of a polymerised fibrin (FBN) network and entrapped platelets. This FBN clot prevents blood loss by filling severed vessels within minutes of trauma. The activation, amplification and inhibition of blood coagulation will be discussed in detail in section 1.1.2.

As well as 'plugging' leaking vessels, the FBN clot provides a temporary scaffold in the wound space for cells to migrate into and repopulate the area. Platelets are intrinsically associated with these events. Following the activation of the coagulation cascade, platelets aggregate at the site of injury and facilitate blood clot formation by interacting with the FBN network. Upon activation at the site of trauma, platelets also secrete multiple cytokines and growth factors that regulate the local cellular activity immediately after injury.

Inflammation and Inflammatory Cells

Blood coagulation is one of the very early phases of tissue repair and it is followed by a variety of reactions involving inflammatory cells. These reactions are collectively termed the *inflammation phase*. Blood coagulation and inflammation are tightly intertwined and cross-regulate each other.

Inflammatory cells are generally present in the circulation in a latent form, and are recruited under the action of chemotactic cytokines to sites of tissue injury where they gain their activated phenotype. However, resident inflammatory cells also exist. They are mainly tissue macrophages as well as some mast cells. The main inflammatory cell types, including neutrophils, monocytes/macrophages and lymphocytes will be discussed briefly in the rough chronological order of their appearance at the wound site during normal tissue repair.

- Neutrophil infiltration

In the initial stages of tissue repair and inflammation, neutrophils (reviewed by Brieland and Fantone, in Phan and Thrall, 1995; by Clark, in Clark, 1996; and by Holland *et al.,* in Crystal *et al.,* 1997), other polymorphonuclear leukocytes cells and monocytic cells start migrating into the injured tissue simultaneously. However, neutrophils become detectable first because of their abundance in the circulation and rapid mobilisation (reviewed by Hogg in Crystal *et al.,* 1997). Neutrophils and monocytes respond to a number of common chemoattractants, including fibrinopeptides and fibrin degradation products, complement factor 5a, platelet-activating factor (PAF) released from endothelial cells as well as PDGF, TGF-β and PAF from platelets.

These growth factors and cytokines up-regulate integrin CD11b/CD18 on the neutrophil surface, allowing adhesion to the activated endothelium and neutrophil transmigration into the tissue. Second, these cytokines stimulate neutrophil elastase and collagenase release which contribute to ECM degradation. One of the main roles of neutrophils is to deplete the injured tissue from necrotic tissue fragments and bacteria by endocytosis. This is mediated by proteolytic, enzymatic and oxygen radical mechanisms.

If wound contamination has not occurred, neutrophil infiltration usually ceases within a few days. Most neutrophils become entrapped in the fibrin clot and desiccated tissue. The remaining neutrophils become senescent, apoptose and are phagocytosed by macrophages.

- Macrophage accumulation

Within days of tissue injury, monocytic cells are recruited from the vasculature to the wound site where they acquire their activated macrophage phenotype and add to the resident tissue macrophage population (reviewed by Shaw and Kelley, in Phan and Thrall, 1995; and by Clark, in Clark, 1996). First, monocytes gain one of three main activated states under the action of the local cytokine network; either *cytocidal, inflammatory* or *repair* phenotype (reviewed by Riches, in Clark, 1996). The responsive macrophages act in concert with neutrophils to phagocytose dead tissue debris and invading pathogenic micro-organisms. As macrophages become established at the wound site, they show a high capacity for phagocytosis, increased levels of lysosomal enzymes and they proceed to engulf and destroy apoptotic neutrophils. This is facilitated by the secretion of a variety of proteases such as plasminogen activator and elastase. In addition to their function in tissue debridement, macrophages regulate tissue regeneration through dynamic interactive processes with surrounding cells via the secretion of soluble cytokines and growth factors (reviewed by Riches, in Clark, 1996; and Bezdiek and Crystal, in Crystal *et al.,* 1997).

Activated platelets release PDGF and TGF-β via degranulation and play a predominant role in the initial recruitment of inflammatory cells into the tissue during early inflammation. However, macrophages and other cell types amplify and sustain growth factor production at the site of injury by secreting more peptides with chemoattractant, mitogenic and other autocrine and paracrine properties. These numerous molecules include PDGF-AB, TGF-β1, HB-EGF, IGF-1, IL-1, IL-6, IL-8, TGF-α and TNF-α (for reviews, see Clark, 1996).

Macrophages play a role in a multitude of events via the release of these various mediators, regulating debridement, ECM deposition and new blood vessel formation. Macrophage mediators target fibroblasts, neutrophils, monocytes and endothelial cells which migrate towards the centre of the wound, synthesise ECM components and proliferate. In particular, macrophages are a major source of TGF-β1, a major regulator of fibroblast ECM secretion that directly enhances connective tissue formations. Thus Macrophages are vital for normal wound healing and if their infiltration is prevented, healing is impaired (Leibovich and Ross, 1975; reviewed by Riches, in Clark, 1996). Finally, it is interesting to notice that if neutrophils do eventually undergo apoptosis *in situ*, the fate of macrophages during the resolution of the inflammatory phase remains totally obscure (reviewed by Haslett and Henson, in Clark *et al.*, 1996).

- Lymphocytes

Lymphocytes are the immune effector cells (reviewed by Richeldi *et al.*, in Crystal *et al.*, 1997). There are two main classes of lymphocytes. B-lymphocyte are responsible for the production of antibodies and humoral immunity, whereas T-lymphocytes regulate cell-mediated host defense. There are two subgroups of T-lymphocytes differentiated by the cell-surface expression of either CD4 (MHC class II receptor) or CD8 (MHC class I receptor) antigens. CD4+ T-lymphocytes are classically named *T-helper* cells and CD8+ T-lymphocytes are known as *cytotoxic* lymphocytes.

Certain lymphocytes in the presence of antigen-presenting cells also have the ability to release growth factors and cytokines that take part in tissue repair processes. These mediators can be separated in two groups according to the nature of the immune response and the predominant involvement of one of two types of activated antigen-specific CD4+ T-helper cells.

In short, type 1 T-helper cells (Th1) express IFN-γ, IL-2, and lymphotoxins and stimulate macrophage activation, lymphocyte proliferation and cell-mediated immune responses. Type 2 T-helper cells (Th2) express IL-4, IL-5, IL-9, IL-10 and IL-13 that are important in antibody-mediated responses, macrophage suppression, anti-helminthic and allergic responses. Other T-helper cells (Th0) have the ability to express both sets of cytokines (Moller, 1999). Distinct

cytokine patterns can be observed in different infectious, allergic and auto-immune diseases, depending on the dose of antigen, its processing and the preliminary presence of cytokines in the environment of the lymphocytes.

- Other inflammatory cells

Basophils, eosinophils and mast cells are granulocytes and exhibit similarities such as prominent intracellular secretory granules.

Mast cells and basophils are major sources of histamine, chemoattractants, proteoglysans, serine proteases and heparin. They play a role in immunological functions and allergy (reviewed by Ruoss and Caughey, in Phan and Thrall, 1995; and by Galli and Costa, in Crystal *et al.,* 1997). Some differences in mast cell and basophil location, role and natural history make them distinct cell types. For instance, mast cells are resident cells that differentiate in tissues whereas mature basophils are found in the circulation. Furthermore, basophils are the main source of blood-borne histamine, but mast cells are the main histamin storing cells in tissues. Mast cell granules also contain ready-made stores of a great variety of cytokines, including the major pro-inflammatory cytokines IL-1 and TNF-α, as well as the inflammatory chemoattractants MCP-1 and MIP-1α and -1β (reviewed by Riches, in Clark, 1996; and by Strieter and Kunkel, in Crystal *et al.*, 1997). It is thought that mast cells may be able to initiate the cytokine cascade and propagate inflammatory cell activation via the release of these molecules. As the reactions progresses, cytokines from mast cells, other resident cells and inflammatory cells exert complex effects on vascular endothelial cells, fibroblasts, epithelial cells and nerves However, the exact contribution of these inflammatory cells is not completely understood.

Eosinophils, like basophils, are polymorphonuclear leukocytes characterised for their strong staining when treated with eosin. Eosinophils are involved in the clearance of parasitic infections and are not present in great numbers during normal tissue repair (reviewed by Ruoss and Caughey, in Phan and Thrall, 1995; and by Allen and Davis, in Crystal *et al.,* 1997). Their presence indicates either parasitic infection or dysregulated inflammatory functions such as in some types of asthma. However, eosinophils can secrete IL-4 and IFN-γ and contribute to the cytokine network. It is thought that in concert with mast cells, eosinophils may play a role in the down-regulation of the inflammatory response via the release of cytokines that are only partly identified.

Granulation Tissue Formation

In the early phases of repair, the injured tissue becomes richly vascularised, giving it a granular appearance. This is referred to as *granulation tissue* (reviewed by Madri *et al.,* in Clark, 1996). The site of trauma becomes densely populated with fibroblasts and macrophages that move along the FBN lattice (reviewed by McCarthy *et al.,* in Clark, 1996). In time, the temporary FBN network is degraded by the fibroblasts, cleared away by inflammatory cells and replaced by a loose newly-synthesised collagen-rich connective tissue that also comprises fibronectin and hyaluronic acid (Grevstad, 1988; reviewed by Yamada and Clark, in Clark, 1996).

As the principal cell type found in connective tissue, fibroblasts play the crucial role of generating the definitive ECM components that replace the FBN clot (diagram 1; reviewed by Tremblay *et al.,* in Phan and Thrall, 1995; and by Desmouliere and Gabbiani, in Clark, 1996). Fibroblasts secrete serine proteases that initiate the degradation of the FBN lattice, such as urokinase-type plasminogen activator and tissue-type plasminogen activator (Hayakawa *et al.,* 1995). In turn, these proteases activate plasminogen into plasmin that degrades the FBN lattice and other ECM components (Vassali *et al.,* 1991). In addition, fibroblasts synthesise, deposit and remodel all the main ECM elements, including fibronectin and collagens.

'Collagens' is a general term that embraces the superfamily of collagenous proteins (reviewed by McAnulty and Laurent, in Phan and Thrall, 1995; and by Chambers and Laurent, in Crystal *et al.,* 1997). Collagens occur as a variety of proteins sharing collagenous motifs and are the principal structural proteins of connective tissue. Amongst their many roles, collagens provide tensile strength to the stroma and a substratum for cell attachment and migration. Collagens can also regulate cell behaviour, growth factor presentation via cell-matrix interactions (Ayad *et al.,* 1998, see section 1.1.2).

The most abundant collagens in connective tissue are the interstitial collagen types I and type III. The ratio of collagen type I to type III varies according to the tissue, but type I is generally predominant in most organs. During tissue repair, collagens and other matrix molecules such as fibronectin and proteoglycans (reviewed by Varani, in Phan and Thrall, 1995) are rapidly synthesised, deposited and degraded to regenerate or replace the compromised stroma. During this phase overall collagen synthesis is increased and more collagen type III than type I is produced by fibroblasts (reviewed by Yamada and Clark, in Clark, 1996).

Tissue Remodelling

As the ECM is accumulated at the site of injury, it forms a changing micro-environment that regulates cell function in a process that has been termed *dynamic reciprocity* (Clark, 1996, for review). Fibroblasts acquire a contractile phenotype and reduce the size of the injured area by contracting the ECM. Progressively, endothelial and inflammatory cells apoptose or leave the area and numerous microvessels disappear in a phase of *resolution* of the healing process. When all the inflammatory cells have left and only mature vessels remain, fibroblasts form the vast majority of the remaining cells and they reorganise the stroma. The ratio of collagen type I to type III produced by fibroblasts increases to resemble that of the normal tissue. Slowly, the fibroblasts modify the nature the composition, quantity and organisation of the ECM. This process of ECM maturation and refinement is often termed *remodelling.*

Over weeks and months, collagens become the predominant ECM proteins. Collagen fibres increase in size, become cross-linked and give tensile strength and resilience to deformation to the scar tissue (Ayad *et al.,* 1998; reviewed in Chambers and Laurent, 1998). Finally, the mature ECM causes fibroblasts to apoptose, leaving a relatively acellular *scar tissue.*

1.1.2 CELL-MATRIX AND CELL-CELL INTERACTIONS

On the surface of cells, there are a number of adhesion and ECM receptor molecules that facilitate cell-cell and cell-matrix interactions. They fall into three main categories: **a)** Those present on the basal surface of cells and function as *cell-matrix* adhesion receptors such as integrins and clusters of differentiation antigen-44 (CD44), **b)** Those that localise to points of *intercellular contacts* such as cadherins, inter-cellular adhesion molecules-1 and -2 (ICAM-1, ICAM-2), **c)** Those that are on the luminal surface of cells that function as *cell-cell adhesion* molecules important in leukocyte- or platelet-endothelial adhesion such as platelet-endothelial cell adhesion molecule-1 (PECAM-1), endothelial-leukocyte adhesion molecule-1 (ELAM-1), and vascular cell adhesion molecule-1 (VCAM-1).

Cell-Matrix Interactions

The extracellular matrix is comprised of a vast array of proteins with varying structural and cell regulatory functions (reviewed by Mutsaers *et al.,* 1997). These components impose structure to their environment, but they are also connected to the cells via specific cell-surface receptors. These ECM receptors conduct information from the ECM network to the intracellular

cytoskeleton. Interactions between the ECM and cells provide cues for growth and differentiation which are governed by the nature of the ECM and the isotype of receptor engaged.

Integrins are the best described ECM adhesion molecules (reviewed by Yamada *et al.,* in Clark, 1996; and Wickham, in Crystal *et al.,* 1997). They form a family of heterodimeric (α/β) molecules, of which there are 8 known β subunits and at least 16 α subunits. However, they only dimerise in about 20 structurally distinct integrins as many α subunits can only associate with particular β subunits. Integrins are found on the surface of most cell types, but each cell expresses a particular group of integrins. The expression of particular integrins is further regulated by the ECM in the cell environment. Their ligands include fibrin, fibrinogen, fibronectin, laminin, various collagens, entactin, tenascin, thrombospondin, vitronectin and von Willebrand factor (reviewed by Yamada, *et al.,* in Clark, 1996).

It is now realised that integrins play other important roles in tissue repair as they mediate cell adhesion, spreading and migration, as well as wound contraction. In addition, integrins have very recently been shown to contribute to growth factor activation. Indeed, integrin $\alpha v\beta 6$ binds latent TGF-β1 and promotes its' activation. Genetically modified animals deficient for this integrin are protected from chemically-induced tissue fibrosis and develop exaggerated inflammation instead (Munger *et al.,* 1999). This suggests that the growth factor activating function of integrins is to be taken into account in tissue repair and in the development of fibrotic diseases.

Cell-Cell Interactions

The principal cell-cell interaction molecules are β1 and β2 integrins in conjunction with E-, L- and P-selectins as well as immunoglobulins ICAM-1, -2 and VCAM-1. In particular, these molecules mediate leukocyte adhesion to endothelial cells (reviewed in Cotran and Mayadas-Norton, 1998). The recognition of endothelial cells surface moieties by leukocytes is essential to their extravasation and influx into injured tissues (reviewed in Zimmerman *et al,* in Crystal *et al.,* 1997). This process can be broken down in several phases that require several molecules including selectins, such as ELAM-1, that are found on the surface of activated endothelial cells. The expression of selectins and the other cell-surface adhesion molecules is itself regulated by inflammatory cytokines coming from the underlying tissue such as IL-1, interferon-γ (IFN-γ) and TNF-α. Platelet aggregation is another critical step of normal tissue repair that is mediated via cell-cell adhesion molecules. Fibrin and fibrinogen bind the main platelet integrin $\alpha_{IIb}\beta_3$ and form bridges between aggregating activated platelets (reviewed in Herrick *et al.,* 1999).

1.1.3 GROWTH FACTORS AND CYTOKINES

Over the last 20 years, a large number of soluble peptides that are released locally by cells have been discovered. These peptides include polypeptide growth factors and cytokines that play key roles in regulating cell and tissue function. These mediators can be produced by cells that govern their own function (*autocrine effect*), or that of other cells (*paracrine effect*).

A large number of these molecules play important roles during tissue repair and host defense mechanisms (reviewed in Moulin, 1995; and Clark, 1996). These molecules can separated in two main groups. They are growth factors that regulate cell movement, replication and biosynthetic function are generally involved in tissue repair mechanisms, and cytokines that initiate and regulate inflammation and defense mechanisms of the body. Most factors play such various functions that they fit with either description, but a general consensus has been adopted for simplicity.

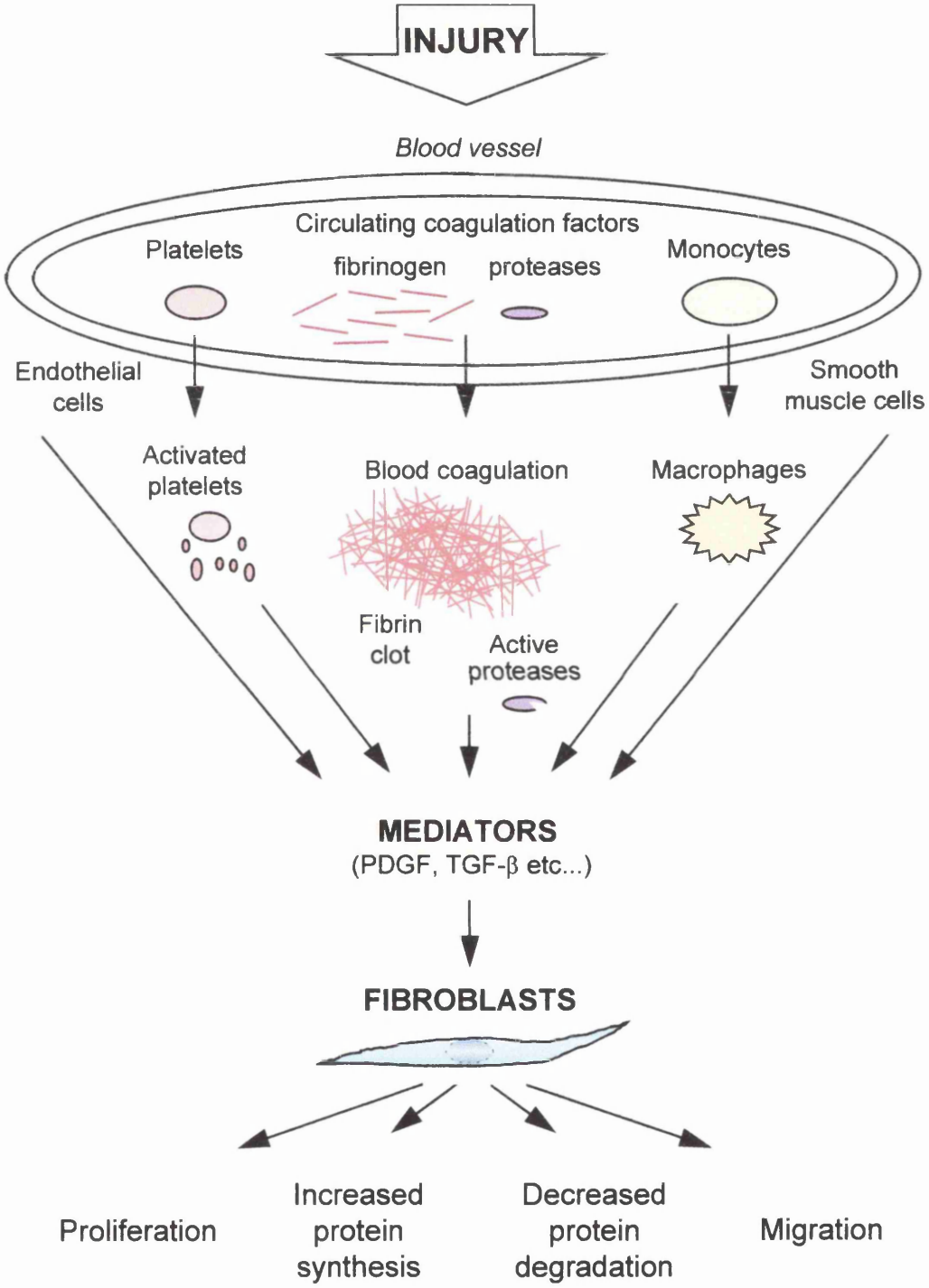
The aim of this section is to acknowledge the great variety of cell function mediators since the majority of them play a role in tissue repair. An overview of these molecules will be given next and a diagram shows the main growth factors and cytokines that regulate fibroblast function (diagram 2, page 32).

Growth Factors in Tissue Repair

A host of growth factors regulate the proliferation and biosynthetic activity fibroblasts and other cells, thereby controlling tissue inflammation and regeneration (diagram 1 page 26, diagram 2 page 32; Clark, 1993; Mutsaers *et al.*, 1997, for review). These growth factors include several families of fibroblast mitogens and profibrotic mediators such as platelet-derived growth factors (PDGF), transforming growth factor-beta isoforms (TGF-β). fibroblast growth factors (FGF), epidermal growth factor-like molecules (EGF), insulin-like growth factors (IGF) and endothelin-1. Many growth factors exist in a number of isoforms with different effects and varying specificity for cell surface receptors, some of which will be discussed below.

Immediately after tissue injury, a number of these growth factors such as PDGF and TGF-β originate mainly from degranulating platelets that function as reservoirs for cytokines. Later, inflammatory cells, and macrophages in particular, propagate inflammation and orchestrate tissue regeneration through *de novo* cytokine production. In addition, subsets of cytokines also secreted by fibroblasts and other tissue cells add to this varied pool of growth factors and cytokines and form a complex regulatory network for cellular activity (Stouffer and Runge, 1998, for review).

Diagram 1 **Mechanisms involved in the regulation of fibroblast activity during tissue repair and fibrosis**



- The platelet-derived growth factor family

The cytokine PDGF is a potent stimulator of DNA synthesis and replication for fibroblasts and smooth muscle cells (reviewed by Betsholtz, in Phan and Thrall, 1995; Hughes *et al.* 1996). The PDGF family consists of dimers of two polypeptide chains, denoted A and B, which form the dimers PDGF-AA, -AB and -BB. Each of these isoforms binds to one or both of the two integral membrane PDGF receptors. PDGF-AA only interacts with PDGF α-receptor, PDGF-BB only with its' PDGF β-receptor whereas PDGF-AB can bind to either receptor type (reviewed in Heldin and Westermark, in Clark, 1996; and Claesson-Welsh, 1996).

The treatment of injured tissues with PDGF-AB or -BB was shown to accelerate repair and stimulate granulation tissue formation in skin wounds (Pierce *et al.*, 1991a; Pierce *et al.*, 1991b). PDGF stimulates tissue repair mainly by recruiting fibroblasts to the site of injury and stimulating their proliferation, but it also increases ECM gene expression, including that of fibronectin and procollagen type I and III (Lepisto *et al.*, 1995; reviewed in Butt *et al.*, 1995). PDGF also stimulates TGF-β1 in fibroblasts *in vitro* and in granulation tissue (Pierce *et al.*, 1989), providing another mechanism for PDGF to up-regulate matrix deposition at the wound site.

- The transforming growth factor-β family

TGF-β occurs in five different isoforms (reviewed by Roberts and Sporn, 1993; by Khalil and O'Connor, in Phan and Thrall, 1995; and by Roberts and Sporn, in Clark, 1996). Although originally identified in platelets, TGF-β1 is also expressed by most mammalian cells, including fibroblasts and smooth muscle cells. TGF-β1 is the most potent cytokine to induce the production of ECM molecules, including collagen and fibronectin. I*n vivo*, the topical application of TGF-β1 increases the tensile strength of dermal wounds (reviewed in O'Kane and Fergusson, 1997). In contrast to the effects of PDGF, the topical application of TGF-β1 in dermal wounds does not stimulate fibroblast infiltration, but it greatly increases the amount of collagen deposited and induces the formation of more mature collagen fibres (Pierce *et al.*, 1991a; Pierce *et al.*, 1991b).

- The fibroblast growth factor family

FGFs were first identified as mitogens for 3T3-mouse fibroblast-like and endothelial cells (reviewed in Abraham and Klagsburn, in Clark 1996). Two forms of FGF with an acidic (acidic FGF or aFGF), or a basic iso-electric focusing point (basic FGF or bFGF), were initially identified. There are now at least nine isoforms of FGF that have been renamed and numbered 1 to 9. The main sources of FGF-2 are damaged endothelial cells and macrophages, but FGFs may

also be produced by several other cell types. FGFs are strongly mitogenic to endothelial cells and one of the main families of growth factors involved in angiogenesis. In addition, FGFs are mitogenic to keratinocytes *in vitro* and promote re-epithelialisation and dermal healing *in vivo*. FGF-1 and -2 have been shown to stimulate granulation tissue formation and increase fibroblast proliferation and collagen deposition

- The endothelial growth factor family

The EGF family of proteins includes transforming growth factor-alpha (TGF-α) and heparin-binding epidermal growth factor (HB-EGF), (reviewed by Nanney and King, in Clark, 1996). TGF-α is found predominantly in development and has sometimes been termed*fetal EGF*, however, it is also found in a restricted number of adult cells such as macrophages and keratinocytes. EGF-like molecules exert direct mitogenic effects on a range of cells including fibroblasts, keratinocytes, smooth muscle cells and epithelial cells. The topical administration of EGF and TGF-α in experimental and clinical settings revealed that they are potent enhancers of epidermal regeneration and tensile strength in dermal wounds. In addition, EGF over-expression leads to enhanced collagen accumulation and up-regulates full thickness wound tensile strength. EGF has also been implicated in various tissue repair events such as liver tissue regeneration and corneal re-epithelialisation.

- Insulin-like growth factors

IGF-1 and -2 are produced principally in the liver and found in the circulation. They share homologies with pro-insulin and exert insulin-like metabolic effects. IGFs are often found associated with one of at least 6 large associated binding proteins (IGF-BP-1 to -6). However, TNF-α can stimulate IGF production in most tissues, including macrophages and fibroblasts. Furthermore, IGF-1 and -2 can activate a great variety of cell types. In particular, IGF-1 is known to stimulate fibroblast replication and ECM production, including collagen, elastin and proteoglycans. Finally, increased levels of IGF levels have been reported in some fibrotic lesions, which may indicate a role in connective tissue deposition and the regulation of local cell growth and differentiation (reviewed by Riches,.in Clark, 1996).

- Endothelin-1

Endothelin-1 was originally discovered as a potent vasoconstrictor, but it is now recognised as a pluripotent mediator that also stimulates fibroblast migration, proliferation and

collagen deposition (Peacock *et al.,* 1992; Dawes *et al.,* 1996). Endothelin-1 originates from endothelial cells, epithelial cells, macrophages and fibroblasts. Endothelin-1 levels are elevated in patients with pulmonary fibrosis and systemic sclerosis. It is a possible contributing factor to tissue fibrosis and constitutes with most of the pre-cited growth factors, a potential target to control connective tissue deposition (reviewed in Mutsaers *et al.,* 1997).

- Connective tissue growth factor

A novel fibroblast growth factor has been discovered more recently and termed connective tissue-growth factor. It is an IGF-BP-related protein and was first discovered in endothelial cells and named IGF-BP-r2 (Bradham *et al.,* 1991). However, CTGF appears to have strong regulatory functions on fibroblasts that are independent from IGF or IGF-BP. TGF-β1 is the only known stimulator of CTGF described to date and CTGF appears to be a critical mediator of the effects of TGF-β1 (reviewed by Grotendorst, 1997). In particular, CTGF stimulates fibroblast proliferation, ECM production and granulation tissue formation (Frazier *et al.* 1996).

Inflammatory cytokines in Tissue Repair

In addition to growth factors, there are a number of other cytokines that come mainly from blood-borne and resident inflammatory cells (reviewed in Remik and DeForge, in Phan and Thrall, 1995). Once secreted, they form a complex regulatory network regulating mainly immune defense mechanisms. However, some of these inflammatory cytokines can be induced in mesenchymal cells and their pluripotent effects also affect tissue repair.

Interleukin-1 (IL-1) was one of the first cytokines discovered. It was detected in the supernatant of activated cultured macrophages as an endogenous polypeptide capable of inducing fever. Tumour-necrosis factor (TNF) was discovered soon after as a macrophage-derived molecule capable of inducing tumour necrosis, hence the term TNF. IL-1 and TNF subsequently served as a prototypes for other inflammatory cytokines such as IL-8, macrophage chemotactic protein-1 (MCP-1) and others. Most were given an interleukin number they were discovered, but some cytokines did not follow that lead. Some of the key cytokines in tissue repair and fibrosis will be discussed next (reviewed by Roitt *et al.,* 1993; and Wewers *et al.*, in Crystal *et al.,* 1997).

- Interleukin-1 and tumour necrosis factor-α

Both IL-1 and TNF exist in α and β isoforms. These four molecules have considerable overlap in their functions. They all have pyrogenic activity and promote fibroblast and endothelial

cell activation. However, IL-1 is more efficiently processed in monocytes, whereas TNF-α is produced principally by macrophages (reviewed by Wewers *et al.*, in Crystal *et al.,* 1997).

IL-1 simulates a myriad of cellular functions on such divergent cell subtypes as fibroblasts, lymphocytes, osteoclasts, skeletal and smooth muscle cells, hepatocytes, neutrophils, epithelial and endothelial cells. The two isoforms of IL-1 overlap in most of their functions.

In susceptible cell types, TNF-α induces apoptosis, cell differentiation and activation, cell surface receptor expression, cytokine release, oxidant and protease secretion. In fibroblasts, IL-1 and TNF stimulate proliferation and collagenase release, thereby effecting directly ECM turnover. In addition, IL-1 and TNF regulate immune functions and play a cofactor role in the cell-cell regulation of B-lymphocytes by T-lymphocytes, in conjunction with interleukins.

However, one of the most profound of the early effects of IL-1 and TNF is the induction of secondary cascade of cytokines. Two of the most recognised cytokines in this cascade are IL-8 and IL-6. In turn, IL-6 and IL-8 contribute to neutrophil and monocyte recruitment and activation, thereby amplifying host defense and tissue repair mechanisms.

- Interleukin -8

IL-8 is a major neutrophil chemoattractant, but it is also chemotactic for eosinophils and some T-lymphocytes. IL-8 is primarily secreted by macrophages, but it can also originate from fibroblasts and endothelial cells in response to IL-1β or TNF-α. IL-8 also stimulates the release of additional chemoattractants such as macrophage-chemotactic protein-1 (MCP-1) that amplify inflammatory cell recruitment and differentiation after tissue injury.

- Interleukin -6

Another major inflammatory cell and macrophage cytokine stimulated by IL-1 and TNF is IL-6. IL-6 up-regulates B-lymphocyte function and antibody production. It also promotes the secretion of acute phase inflammatory proteins by hepatocytes, including fibrinogen and other coagulation cascade factors. It is now accepted that IL-1 and TNF are the primary inducers of IL-6 during inflammation. This is consistent with the fact that IL-6 reaches a peak after about 4 hr to 8 hr after infection, whereas IL-1 and TNF peak after about 90 min.

- Interferon-γ and interleukin -10

Most inflammatory cytokines are know to promote inflammation and tissue repair. However, there are instances of cytokines that have some anti-inflammatory functions in addition

to their role in host defense mechanisms, such as anti-parasital functions. Interferon-γ (IFN-γ) and IL-10 are the most recognised of these cytokines (reviewed by Roitt *et al.,* 1993; and by Rovatti*et al.,* in Crystal *et al.,* 1996).

IFN-γ is expressed mostly by lymphocytes, including Th1 T-lymphocytes, as well as epithelial cells and fibroblasts. It targets a great variety of tissue cells and leukocytes, including Th2 cells. IFN-γ acts as a primer for monocyte to macrophage differentiation and stimulates macrophage activation. It promotes MHC class II molecule expression, lymphocyte-endothelial cells adhesion by up-regulating cell adhesion receptor expression. Finally, IFN-γ down-regulates overall cytokine production, which has anti-inflammmatory effects.

IL-10 is the second best-described cytokine with anti-inflammatory properties. IL-10 is secreted by Th2 T-lymphocytes and targets mostly Th1 T-lymphocytes. IL-10 down-regulates MHC class II expression in monocytes and its inhibitory effects on cytokine production in leukocytes dampen immune and inflammatory processes.

- Other cytokines

A number of additional interleukins have been discovered, including IL-2 to -5, IL-7, IL-9 and IL-11 to -13. Although some of these interleukins affect particular mesenchymal cell functions, they are mostly relevant to the T- and B-lymphocyte cross-talk in the regulation of antibody- and cell-regulated immunity. These effects are somewhat removed from tissue repair and fibrosis (their functions are reviewed by Feldman, in Roitt *et al.,* 1993).

The variety of inflammatory cell chemoattractants, or *chemokines*, and the redundancy in their functions should also be acknowledged to represent more fairly the wide array of existing cytokines (reviewed in Kunkel *et al.*, in Phan and Thrall, 1995). Granulocyte and macrophage colony-stimulating factors (G-CSF, M-CSF, GM-CSF), and macrophage chemotactic factors (MCPs) are only a few of the many chemokines that mediate leukocyte adhesion, recruitment, differentiation and function during tissue repair and fibrosis.

Summary

In summary, there is a myriad of growth factors and cytokines that stimulate inflammatory and mesenchymal cells directly or by inducing the production of other mediators. These molecules form a complex network of regulatory interactions and control connective tissue formation by fibroblasts and other tissue cells. In normal tissue repair, the balance reached between the effects of these many cytokines and the normal resolution of the inflammatory phase

allow complete coherent healing. However, the dysregulation of the cytokine network may result in tissue fibrosis and threaten the functionality of the damaged organ.

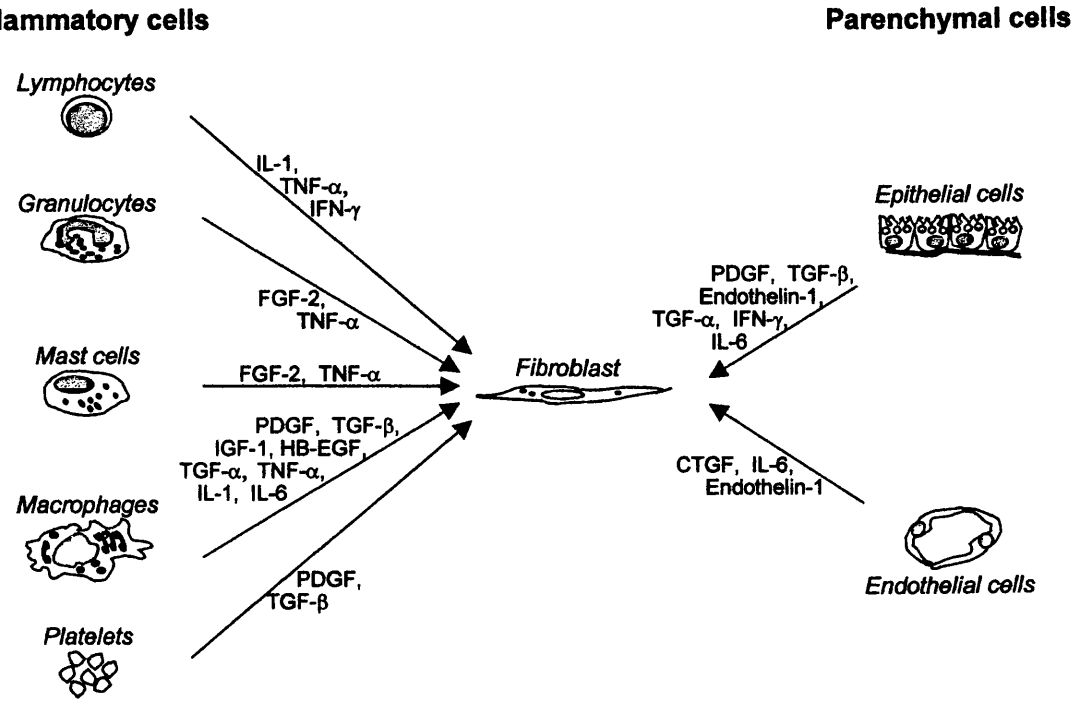


Diagram 2 **Some of the cytokines and growth factors that regulate fibroblast functions.** Fibroblast functions are regulated by a variety of paracrine cytokines and growth factors. These originate from resident and circulatory inflammatory cells such as macrophages, as well as tissue cells, including epithelial and endothelial cells.

1.1.4 TISSUE FIBROSIS

Excessive ECM accumulation following tissue injury can disrupt further the architecture of the damaged organ and form the basis of *fibrotic diseases*, a heterogeneous group of disorders with similar histopathological features. Their development is characterised by excessive cell proliferation and deposition of ECM. These events are generally reminiscent of normal tissue repair and collectively termed *tissue fibrosis* (diagram 1; reviewed by Mutsaers *et al.*, 1997).

The current hypothesis proposes that tissue fibrosis may develop after an initial insult triggers an inflammatory and fibroproliferative response similar to that observed during the early

phases of normal tissue repair. Cells and blood-borne proteins may infiltrate the interstitium of the affected area in response to injury. Subsequently, growth factors from the blood and activated inflammatory cells could stimulate fibroblasts and other collagen-producing cells to migrate, proliferate and produce excess collagen, resulting in tissue fibrosis (Marshall *et al.*, 1997, for review; Meduri *et al.,* 1998). This combination of excessive ECM synthesis, sustained fibroblast proliferation and decreased ECM degradation results in the formation of connective tissue which gradually overwhelms the fibrosing tissue and compromises its' function. Furthermore, tissue fibrosis is an on-going progressive event characterised by the sustained formation of connective tissue, suggesting that fibrosis arises from a continuous stimulus (Kuhn *et al.,* 1989).

There are observations that question the importance of the contribution of inflammatory cells in the establishment of tissue fibrosis. For instance, procollagen processing and deposition can be observed principally in fibrotic lungs of patients with ARDS after injury. The extremely short time between tissue damage and setting of fibrosis, signalled by elevated procollagen processing and deposition, seem to suggest that tissues can respond to injury by a fibrotic response within hours and without the usual inflammatory phase (Marshall *et al.,* 1998).

The Pathogenesis of Tissue Fibrosis

Tissue fibrosis is generally initiated by some form of tissue injury and arises from different etiologies and precipitants (reviewed by Kuhn III, in Phan and Thrall, 1995). **a)** Connective tissue disease and vasculitic disorders including those of autoimmune origin, such as rheumatoid arthritis, systemic sclerosis, systemic lupus erythematosus, liver cirrhosis and renal sclerosis. **b)** Occupational exposure to chemicals, particles asbestos, silica, paraquat or carbon tetrachloride. **c)** Idiopathic origin linked to trauma and multi-organ dysfunction, such as cryptogenic fibrosing alveolitis, adult respiratory distress syndrome (ARDS) and infantile respiratory distress syndrome (IRDS). **d)** The stimulation of local factors by physical forces, such as systemic hypertension leading to left ventricular hypertrophy, or pulmonary fibrosis triggered by mechanical ventilation. Finally, some types of fibrotic diseases clearly have a familial origin and genetic predisposition may contribute or lead to fibrosis and explain why not all people subjected to profibrotic factors develop tissue fibrosis. There have been a number of studies investigating this possible genetic link, but these studies have yielded poor and conflicting results (reviewed in Marshall *et al.*, 1997). In summary, tissue fibrosis can affect the lungs, heart, liver and kidneys, but the precise mechanisms involved remain mostly unidentified.

Growth Factors and Cytokines in Tissue Fibrosis

Our knowledge of the pathogenesis of tissue fibrosis and the precise mechanisms involved is incomplete, but the resemblance between ECM deposition in normal tissue repair and fibrotic diseases suggests that both responses are mediated by common mechanisms (reviewed in Remik and DeForge, in Phan and Thrall, 1995). The growth factors PDGF and TGF-β1 have been implicated as potential mediators of fibrotic disorders such as interstitial lung disease (Silver, 1995; Silver, 1996; Vaillant, 1996) and liver cirrhosis (Schuppan, 1995). Furthermore, the over-expression of TGF-β1 and PDGF in the lungs in animal models causes fibroblast proliferation and collagen fibril deposition in a fashion reminiscent of a fibrotic disease (Hayashi *et al.*, 1996), suggesting that they may be important in fibrosis. Nevertheless, their precise role and the extent of their contribution remain elusive (reviewed by Cotton *et al.*, 1998).

Current Treatments for Tissue Fibrosis

The current recommended therapies for tissue fibrosis are centered mainly around *immunosuppressive drugs* called corticosteroïds (reviewed by Meyer and Raghu, in Phan and Thrall, 1995). Steroids such as prednisolone mimic endogenous glucocorticoids from the adrenal medulla and have been the mainstay of treatment for over ten years. These drugs have little specificity and target a myriad of inflammatory processes, as well as ECM metabolism to various degrees. However, they are generally inadequate and provide limited benefits to patients. Thus, prognosis for fibrotic conditions remains very poor and in the absence of any specific treatment to down-regulate ECM deposition, there is an urgent need to define the key molecules and cell types that mediate tissue fibrosis (reviewed by Franklin, 1997).

A range of other immunosuppressive drugs, including cyclosporin, azathioprine and cyclophosphamide, has been developed and used alone or in combination with steroïds to improve treatments. Some additional benefits have been reported, but there are many significant side effects using any of these novel immunosuppressive agents.

Novel Potential Treatments

Following extensive *in vitro* and *in vivo* assessment, a number of alternative therapies have been suggested. These drugs were developed to target more specific events that are critical to the development of tissue fibrosis, such as collagen metabolism or cytokine inhibition. Some of these agents are under full clinical investigation and some examples will be discussed next.

- Inhibition of collagen metabolism

Collagen is the main ECM component deposited during tissue fibrosis. Thus, drugs have been designed to inhibit its metabolism at the levels of procollagen gene transcription, mRNA translation and collagen maturation.

In the last year, a study using the drug pirfinidone that inhibits procollagen gene expression in animal models has been shown in an open label study to improve symptoms in patients with pulmonary fibrosis. Furthermore, drugs that block microtubule assembly, such as colchicine, or procollagen processing, such as D-penicillamine, have been reported to alleviate the symptoms of pulmonary fibrosis in small clinical studies (Selman *et al.,* 1998). In addition, proline analogues that disrupt procollagen mRNA translation have been shown to down-regulate ECM deposition in experimental animal models of pulmonary fibrosis (Greco *et al.,* 1997). Finally, inhibitors of prolyl-hydroxylase prevent the formation of stable collagen molecules and improve the outcome of liver fibrosis in animal models (Bickel *et al.,* 1998).

In summary, drugs that target collagen metabolism at various levels have proven beneficial for the symptoms of tissue fibrosis. However, none of the agents tried to date have drastically improved survival rates for lethal fibrotic disorders.

- Targetting growth factors and cytokines

I*n vitro* and *in vivo* studies have shown that there is a wealth of specific fibrotic and inflammatory mediators that could be excellent therapeutic targets for tissue fibrosis. Two main approaches have been considered: One is the delivery of anti-inflammatory cytokines and the other is the inhibition of profibrotic growth factors.

The results of the most potentially important clinical trials in the field of pulmonary fibrosis have recently been reported (Ziesche *et al.,* 1999). This trial evaluated the anti-fibrotic effects of IFN-γ1b and prednisolone in combination and showed that there were substantial improvements in the conditions of patients that failed to respond to normal steroid treatments. These encouraging results confirm that specific cytokine treatments are a viable strategy.

In addition, a general growth factor inhibitor, suramin, has been successfully evaluated clinically to inhibit fibrotic tissue repair locally after eye surgery (Mietz *et al.,* 1998). Suranim blocks the effects of numerous growth factors and it is regarded as another possible alternative treatment for tissue fibrosis. However, the mode of action of suramin remains obscure.

Finally, the specific inhibition of potent inflammatory mediators such as TNF-α and TGF-β1 using specific neutralizing antibodies has also been attempted with some success to down-

regulate pulmonary fibrosis or peritoneal adhesions in animals (Giri *et al.*, 1993; Kaidi *et al.*, 1995). This illustrates the variety of approaches that have been investigated to identify novel therapeutic strategies with greater efficiency and less side effects.

1.1.5 Coagulation Cascade Peptides in Tissue Repair and Fibrosis

Coagulation Cascade Peptides in Tissue Repair

After tissue injury and normal blood clotting, activated coagulation factors and derived peptides are present in tissues. The peptides generated during blood coagulation and the circumstances of their activation will be discussed in detail in section 1.2.

During normal tissue repair, blood coagulation occurs to ensure haemostasis and the amounts of deposited fibrin or activated coagulation cascade factors proceed to decrease over time. For instance, fibrin is degraded and nearly totally disappear from a cutaneous wound within 7 to 10 days of trauma (reviewed in Clark *et al.*, 1996). This implies a tight regulation of blood coagulation processes in space and time. However, high levels of activated coagulation cascade proteases and persistent fibrin deposition are the clinical symptoms of numerous fibrotic diseases and it is possible that coagulation cascade peptides contribute to the development of tissue fibrosis.

Coagulation Cascade Peptides in Tissue Fibrosis

Similar to wound healing, the extravasation of blood-borne coagulation factors and FBN deposition are major features of most fibrotic disorders. FBN deposition is initiated in the early phases of fibrosis and seems to persist throughout the progression of fibrosis (Idell *et al.*, 1991). It has been speculated that fibrin and coagulation factors may regulate connective tissue formation. However, it is not clear whether activation of the coagulation cascade contribute to the disease, or merely a consequence of excessive connective tissue formation and injury to the vasculature.

The coagulation protease thrombin is mitogenic and chemotactic to a wide variety of cells, including fibroblasts (Dawes *et al.*, 1993; Ohba *et al.*, 1994; Ohba *et al.*, 1996). More recently, thrombin has also been shown to up-regulate fibroblast procollagen production *in vitro* (Chambers *et al.*, 1998). These and other cellular effects of thrombin (discussed in detail in chapter four) make this protease a suitable candidate as a mediator of tissue fibrosis.

Evidence of elevated and sustained levels of thrombin activity have been found in a number of fibroproliferative disorders. These include lung fibrosis associated with systemic sclerosis (Ohba *et al.*, 1994; Hernandez-Rodriguez *et al.*, 1995), restenosis following balloon

angioplasty (Wilcox, 1994), renal glomerulonephritis (Sraer *et al.*, 1996) and ARDS (Idell *et al.*, 1987; Idell *et al.*, 1991). The evidence for elevated levels of procoagulant activity and FBN deposition is particularly compelling in the case of pulmonary fibrosis, ARDS and IRDS. This procoagulant activity has been associated with elevated levels of tissue factor and factor VIIa expressed at sites of tissue injury (Imokawa *et al.*, 1997). This means that the extrinsic cascade of the coagulation pathway is activated in these disorders and that it is the main source of thrombin and factor Xa. Bronchoalveolar lavage fluid collected from the lungs of patients with pulmonary fibrosis associated with systemic sclerosis (SSc) are known to be highly proliferative to fibroblasts and a great proportion of this activity (>70%) is attributed to thrombin (Hernandez-Rodriguez *et al.*, 1995). Abnormalities of FBN turnover and elevated levels of procoagulant activity have also been characterised in sarcoidosis and idiopathic pulmonary fibrosis (IPF), (Chapman *et al.*, 1986). Increased levels of extrinsic coagulation pathway activation and tissue factor expression have also been reported in cryptogenic fibrosing alveolitis (Fijii *et al.*, 1998).

In addition, FBN deposition into the alveolar space is characteristic of animal models of pulmonary fibrosis following lung injury (Adamson, 1976) and thrombin activity is elevated in bronchoalveolar lavage fluid in animal models such as bleomycin-induced pulmonary fibrosis in rodents (Tani *et al.*, 1991; Yasui *et al.*, 1998). The concentration of thrombin in these fluids rises until 6 days after instillation of the profibrotic drug, when it reaches its' peak. Furthermore, treatment of the animals with thrombin inhibitors such as antithrombin III and alpha1-antiprotease has been shown to partly ameliorate the fibrotic response (Tani *et al.*, 1991).

Taken together, this evidence supports the idea that thrombin could play a key role in the formation of connective tissue during normal healing and fibrosis.

1.2 BLOOD COAGULATION

The coagulation cascade is a haemostatic mechanism initiated when vessels are damaged, causing blood to leave the vascular compartment and spilling into the neighbouring tissue. This ensures the formation of a provisional FBN clot to plug injured vessels and prevent further blood loss. Successive steps of protease activation occur after tissue injury and follow distinct pathways. These lead to the formation of thrombin and the deposition of FBN. These mechanisms will be reviewed in the following sections and a schematic diagram of the coagulation cascade pathways is represented below. Please note that only the procoagulant events and positive feedback mechanisms are illustrated in this diagram for clarity (diagram 2, page 32).

1.2.1 COAGULATION CASCADE PATHWAYS

The conversion of circulating zymogens into active serine proteases is divided into a number of steps of cell membrane-based binding and limited proteolysis organised in three pathways (Davie *et al.*, 1991); the *extrinsic*, *intrinsic* and *common* pathways (diagram 2).

Activation of the Extrinsic Coagulation Pathway

The extrinsic pathway is initiated on the surface of activated platelets and cells that express the protease receptor tissue factor on their surface either constitutively (e.g. fibroblasts, smooth muscle cells and mesothelial cells), or after activation following damage to blood vessels (e.g. endothelial cells). *In vitro*, fibroblasts and smooth muscle cells are more efficient activators of the extrinsic cascade than resting or activated endothelial cells (Brinkman *et al.*, 1994). Thus, connective tissue cells are as good promoters of blood coagulation as vascular cells.

A very small amount of circulating factor VIIa is sufficient to bind to tissue factor and form a complex in association with cell surface phospholipids and calcium ions (Ca^{2+}). This complex, whose main purpose is to promote the activation of factor X, is termed *tenase* and cleaves the zymogen factor X (fX) into its' active form, factor Xa (fXa). The tenase complex also serves to activate a limited amount of factor IX. At this point, the extrinsic coagulation cascade feeds into the common pathway described in the next sections. However, these limited amounts of fXa and factor IXa play distinct physiologic roles in promoting blood coagulation (Hoffman *et al.*, 1995). The initial amount of tissue factor/factor VIIa-generated fXa appears to be important in initiating platelet activation by generating small amounts of thrombin in the vicinity of the platelets. Thrombin can then act on platelet surface receptors and stimulate platelet aggregation and degranulation. In contrast, the initial amount of factor IXa seems predominantly involved in promoting the generation of fXa leading to the subsequent *prothrombinase* assembly on the platelet surface and the formation of greater levels of thrombin.

Activation of the Intrinsic Coagulation Pathway

The alternative pathway of activation of the coagulation cascade, or intrinsic pathway is triggered when blood comes into contact with a artificial or extravascular negatively-charged substances such as collagen or glass. The intrinsic pathway encompasses the sequential activation of factor XII, XI and IX. It is classically recognised that the initial activation step is mediated by the auto-activation of factor XII on these negatively charged surfaces (Rojkjar and Schousboe,

1997; Citarella *et al.*, 1997). The generation of factor XIIa is thought to result in the activation of prekallikrein into kallikrein and initiation of the kallikrein-kinin system (Motta *et al.*, 1998; Schmaier, 1999, for review). However, recent studies have suggested that the kalllikrein-kinin system can also be triggered on cell membranes as plasma prekallikrein is activated by a membrane-associated cysteine protease on endothelial cells (Rojkjar and Schmaier, 1999). In addition, the intrinsic cascade can also be triggered by a range of cell types with varying efficiency (McGee *et al.*, 1992, for review), including monocytes and macrophages (McGee *et al.*, 1991).

Factor XIIa activates both factor XI into factor XIa and prekallikrein into kallikrein on the surface of endothelial cells (Wiggins *et al.*, 1977) and thereby initiates both the coagulation and the fibrinolytic systems (ten Cate *et al.*, 1996). Eventually, factor XIa activates factor IX into factor IXa (Wolberg *et al.*, 1997). Furthermore, thrombin activates factor XI and activates the intrinsic coagulation pathway (Oliver *et al.*, 1999).

Once activated, factor IXa associates with its' cofactor, factor VIIIa, as well as phospholipids and Ca^{2+} (Lenting *et al.*, 1996). In this macromolecular complex, the proteolytic activity of factor IXa is greatly enhanced and the protease cleaves fX into fXa (Scandura *et al.*, 1996). At this point, the intrinsic and the extrinsic coagulation pathways merge to trigger a common set of reactions in the common pathway.

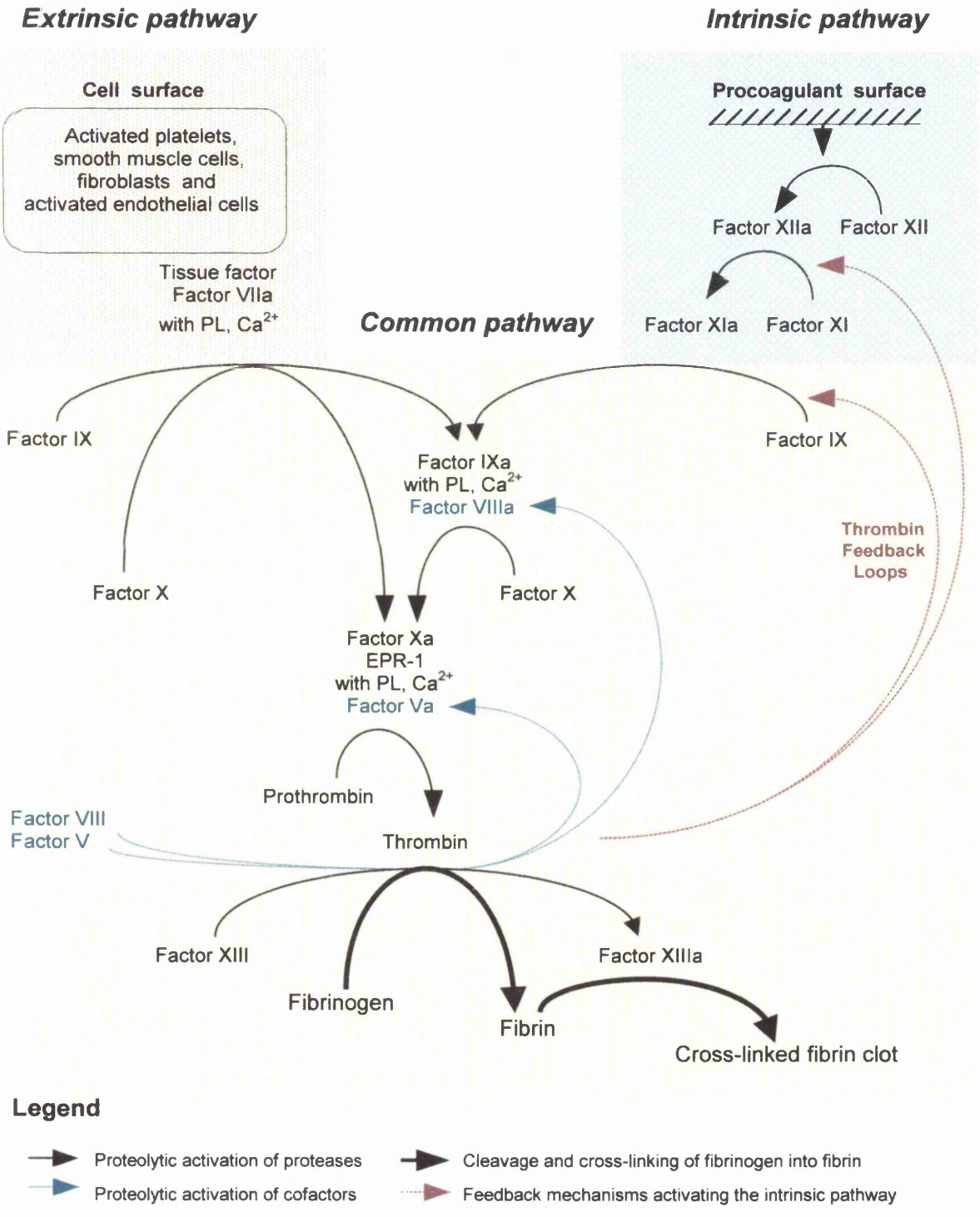
Activation of the Common Coagulation Pathway

Both the intrinsic and the extrinsic coagulation cascade pathways lead to the generation of fXa. In turn, this protease that triggers the common pathway. FXa binds effector cell protease receptor-1 (EPR-1) with high affinity on the surface of platelets (Bouchard *et al.*, 1997) and other cells, such as monocytes (Altieri and Edgington, 1989), leukocytes (Altieri and Edgington, 1990), endothelial cells (Bono *et al.*, 1997a), fibroblasts (Kumar *et al.*, 1995) and smooth muscle cells (Nicholson *et al.*, 1996; Bono *et al.*, 1997a).

EPR-1 was originally described as a factor Va light chain-related antigen on the cell surface of monocytes (Altieri and Edgington, 1989). It was subsequently described as a constitutively expressed membrane form of factor Va in lymphocytes and monocytes (Altieri and Edgington, 1990). The role of EPR-1 was further recognised in mediating the procoagulant effects of fXa, possibly by acting as a cofactor for the protease or by providing high affinity binding sites on the cell surface (Bono *et al.*, 1997).

Diagram 3 **Activation pathways of the coagulation cascade**

The catalytic activity of most coagulation proteases is regulated by their binding to cell surface phospholipids (PL) and the presence of calcium ions (Ca^{2+}). In addition, factors IXa and Xa only gain their full activity when complexed with their respective cofactors, factor VIIIa and Va. Finally, thrombin activates the intrinsic pathway via positive feedback mechanisms.



It has been suggested that EPR-1 may play a crucial role in the cell surface assembly of the prothrombinase complex (Bouchard *et al.*, 1997). Consistent with this idea is the observation that synthetic phospholipid vesicles fail to promote prothrombinase assembly to the same degree as EPR-1-expressing cells such as platelets (Larson *et al.*, 1998). Thus, the prothrombinase complex is formed around fXa bound to EPR-1 on the cell surface and in association with factor Va, phospholipids and Ca^{2+} (Bouchard *et al.*, 1997). Its' role is to convert prothrombin (factor II) into thrombin (factor IIa).

As the final protease to be activated during coagulation, thrombin performs its' procoagulant function by activating platelets, inducing their aggregation and degranulation, and by cleaving four small peptides of fibrinogen (FGN) molecules to convert it into fibrin (FBN). Finally, these FBN monomers polymerise spontaneously into a mesh. This immature form of FBN is then crosslinked by factor XIIIa into an insoluble scaffold that entraps platelets, plugs vessels and eventually fills the wound space.

Distinct Roles of the Coagulation Pathways

With regard to the respective role of the two coagulation pathways, it has been suggested that the extrinsic pathway initiates coagulation immediately after damage to blood vessels and functions to provide an rapid but limited burst of factor Xa and thrombin. These limited amounts of fXa and thrombin are sufficient to induce various feedback mechanisms which amplify the initial signal (reviewed in Davie *et al.*, 1991). Thrombin stimulates local platelet aggregation and triggers the intrinsic pathway, at least in part through the activation of factor IX and XI on the surface of platelets and in the absence of factor XII (Naito *et al.*, 1991; Oliver *et al.*, 1999). Although thrombin only activates trace amounts of factor IX and XI in near-physiological conditions (von dem Borne *et al.*, 1994), these small amounts of factor IXa and XIa contribute to the formation of large amounts of fXa by the dramatic amplification of the coagulation cascade. This in turn results in the sustained generation of thrombin and clot formation (reviewed in Davie, 1995). Furthermore, thrombin activates factor V and VIII that act as cofactors for factor Xa and IXa respectively. These cofactors are critical in conferring their full activity to factor Xa and IXa (reviewed in Hertzberg, 1994). Finally, thrombin and fXa both remain associated to the FBN matrix where they are protected from inactivation by serine protease inhibitors such as antithrombin and heparin. However, fXa is the predominant mediator of the procoagulant activity of formed FBN clots (Eisenberg *et al.*,1993; Prager *et al.*,1995).

In summary, the extrinsic pathway is important in setting up the initial signal for blood coagulation, whereas the intrinsic pathway comprises all the elements of an amplification mechanism that stimulates blood coagulation in full and generates concentrations of thrombin of physiological significance.

Alternative Activation Mechanisms

Because of their particular role and position at the convergence of both coagulation pathways, the activation of factor X and prothrombin is an obligatory step for normal blood coagulation. Therefore, it can be assumed that these factors are activated at sites of FBN deposition. Nevertheless, coagulation proteases can be activated independently of either coagulation pathways and the variety of these alternative mechanisms warrants discussion.

During inflammation, factor X can bind to integrin CD11b/CD18 (also known as MAC-1) on the surface of monocytic cells. Upon stimulation, these cells secrete cathepsin G that cleaves and converts factor X into fXa (Plescia *et al.*, 1996). Because monocytes express EPR-1, they also support prothrombinase formation and prothrombin activation (Altieri and Edgington, 1989).

Coagulation proteases can also be activated during infection by invading organisms. Viruses such as cytomegalovirus, *herpes simplex* virus types 1 and 2 all display the appropriate phospholipids to promote fXa generation in a factor VIIa-dependent, but tissue factor-independent fashion (Sutherland *et al.*, 1997). In addition, bacterial proteinases such as gingipain-Rs from *Porphyromonas gingivalis* cleave and directly activate factor X into fXa (Imamura *et al.*, 1997).

Inhibitory Mechanisms

The coagulation cascade is controlled by negative feedback mechanisms following thrombin generation as well as by circulating and locally produced protease inhibitors.

Thrombomodulin is an integral membrane molecule expressed on the surface of endothelial cells which forms a complex with thrombin (Sadler, 1997, for review). Thrombomodulin alters the activity and affinity of thrombin and in effect, the interaction has the dramatic outcome of switching thrombin from a procoagulant into an anticoagulant factor. In contrast to its' role during coagulation, thrombin bound to thrombomodulin does not catalyse clot formation, but preferentially activates protein C and protein S, which has the opposite effect of down-regulating FBN deposition. Activated protein C (APC) is a serine protease that catalyses the inactivation of factor Va and factor VIIIa through limited proteolysis (Esmon *et al.*, 1997). This reaction is greatly enhanced by phospholipids, Ca^{2+} and activated protein S (APS) which acts as

its' cofactor. The lack of factor Va and VIIIa down-regulates two crucial steps of the coagulation cascade, the prothrombinase and tenase complexes. In this manner, APC and APS control the formation of thrombin through the trigger mechanism (extrinsic pathway) and the generation of additional thrombin brought about by positive feedback mechanisms (intrinsic pathway).

The coagulation cascade is also strongly downregulated by protease inhibitors. Tissue factor pathway inhibitor (TFPI) inactivates factor Xa, forms an inactive binary complex which down-regulates factor VIIa as well as tissue factor activity and blocks the initiation of the coagulation cascade (Lindhal, 1997). TFPI is one of the principal inhibitors of blood coagulation. In contrast, antithrombin III (ATIII), alpha2-macroglobulin (α2-M) and alpha1-antiprotease (α1-AP) inhibit activated proteases. These antiproteases inhibit a wide spectrum of serine proteases, and inactivate the catalytic site of thrombin, factor IXa, Xa and XIa (reviewed in Davie *et al.*, 1995). ATIII is considered the prevailing inhibitor of the coagulation cascade (Fuchs *et al.*, 1983; Fuchs *et al.*, 1984) and its' rate of inhibition by ATIII is increased to various degrees by the presence of heparins. In addition to their inhibitory role during blood coagulation, ATIII, TFPI and α2-M also play a role the catabolism of the inhibited proteases. Within minutes of their inhibition, the antiprotease-protease complexes are cleared from the blood and targeted to the liver where they are finally degraded (Narita *et al.*, 1998; Ho *et al.*, 1996).

Summary

The stepwise activation of serine proteases is central to blood coagulation. This cascade of protease activation is comprised of several pathways that are activated, amplified and inactivated following trauma, fibrotic diseases and infections.

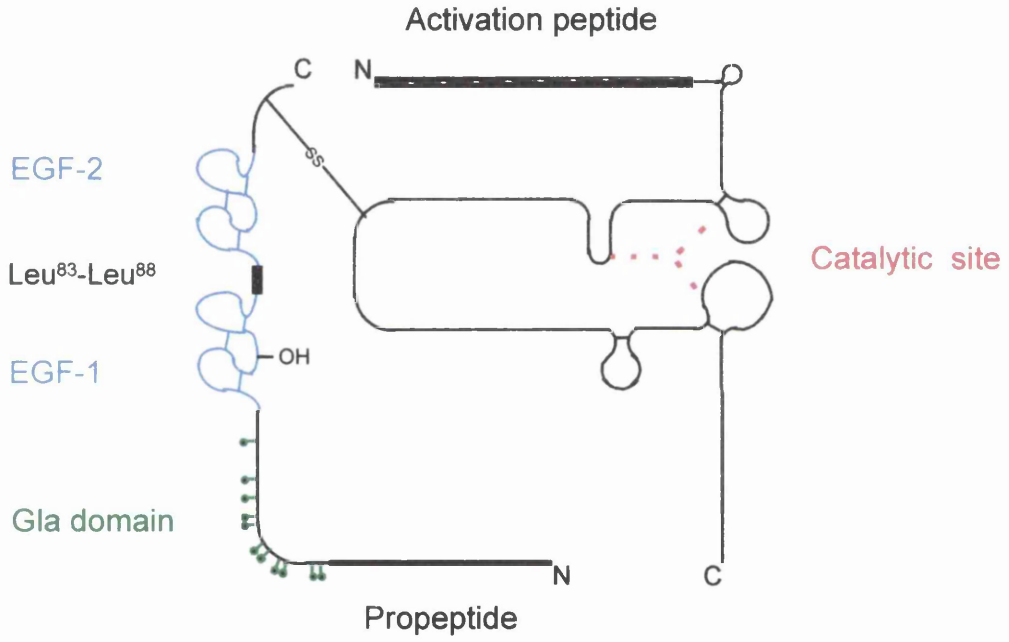
1.2.2 COAGULATION CASCADE PROTEASES

Structural Homologies of the Vitamin K-Dependent Proteins

Prothrombin, factors VII, IX, X, protein C and protein S belong to the family of vitamin K-dependent glycoproteins. They display extensive sequence homology and a similar domain organisation. Each protein also possesses unique structural attributes that determine its' specific function (Davie *et al.*, 1991). A schematic illustration of factor X is shown as a prototypic example of the domain organisation of vitamin K-dependent proteins (diagram 4).

Diagram 4 **Schematic representation of the structure of factor X**

Coagulation factor X comprises most of the domains shared between vitamin K-dependent proteases. This includes a catalytic site formed by the amino acid triad serine-histidine-aspartic acid (doted line), two EGF repeats that regulate protease-cofactor interactions and a Gla domain that binds Ca2+ and phospholipids. These bindings regulate the conformation of the protease as well as protein-protein interactions, In addition, factor X contains a region (Leucine^{83}-leucine^{88}) between the EGF repast that binds to cell surface EPR-1 and promotes prothrombinase activity.



Propeptide	Peptide removed in hepatocytes during zymogen maturation
Gla domain	Cluster of g-carboxyglutamic acid (Gla) residues
EGF-1 & EGF-2	Tandem repeats homologous to epidermal growth factor
Leu^{83}-Leu^{88}	Region binding to the integral cell membrane receptor EPR-1
Activation peptide	Glycopeptide removed upon activation into factor Xa
Catalytic site	Residues forming the catalytic triad

Vitamin K-dependent glycoproteins are produced by hepatocytes in the liver. The mature proteins consist of a single chain when they are secreted into the blood, except for factor X and protein C which consist of two chains which remain linked by a single disulphide bond after endoproteolytic processing. One is termed the *light chain* and the second, which contains a catalytic site, is known as *heavy chain.*

All vitamin K-dependent proteins undergo γ-carboxylation of certain glutamic residues in the endoplasmic reticulum of hepatocytes. This reaction involves vitamin K as an essential cofactor. Each vitamin K-dependent protein contains 9 to 12 γ-carboxyglutamic residues, organised into a cluster in the amino-terminal portion of the proteins called the *Gla domain.* These Gla domains bind Ca^{2+} and other divalent cations and play a role in binding to membrane phospholipids. The binding of Ca^{2+} to the Gla domain regulates both the conformation and the activity of the proteases. In addition, other important features are shared between subsets of these vitamin K-dependent proteins, as explained below.

Coagulation factors II, VII, IX, X and protein C have a serine protease domain similar to pancreatic trypsin. This catalytic site consists of three residues (serine, histidine and aspartic acid) forming a charge-relay system termed a *catalytic triad.* The catalytic triad is the active domain that mediates the proteolytic activation or inactivation of coagulation proteins during clotting.

The light chains of coagulation factors VII, IX, X and protein C contain two tandem repeats homologous to epidermal growth factor, termed *EGF repeats.* Their predominant function is to allow interactions between the proteases and their cofactors and to facilitate substrate recognition. In vitamin K-dependent proteins, the EGF repeats have also been implicated in Ca^{2+} binding and the regulation of protein conformation. However, these strong similarities in domain organisation do not always apply to all vitamin K-dependent proteins, as explained next.

The Catalytic Sites of the Vitamin K-Dependent Proteases

Prothrombin and protein S present unique structural features that may reflect some important differences in their biological function and mechanism of action.

Prothrombin does not display the EGF-like repeats of the light chains of all other coagulation proteases. Instead, two kringle domains are found at the corresponding locations. Furthermore, the light chain of prothrombin is cleaved off upon activation and the active protease performs its' function with only the heavy chain. Indeed, thrombin is fully active even when it is

separated from the procoagulant surface on which it was activated. In contrast, factors VIIa, IXa and Xa retain their light chain after activation which remains connected to the heavy chain by disulphide bonds. Retaining their light chain enables these proteins to establish the interactions with cell membrane phospholipids and cofactors necessary to their function and full catalytic activity.

Protein S is the only protein of the family not to possess a catalytic site, and it does not require minor proteolysis before participating in the coagulation cascade as a cofactor for protein C. Protein S contains four EGF repeats in its' light chain while all other vitamin K-dependent proteins only contain two such repeats, except prothrombin. In addition to these differences in the domain organisation, the other vitamin K-dependent proteins show more discrete variations in amino acid sequence, as reviewed in the next section.

Substrate Specificity of the Vitamin K-Dependent Proteases

The substrate specificity of each coagulation protease is regulated by individual differences in structure at specific locations with which substrates interact for proteolysis to occur. These regions have been termed *specificity pockets* (Resaie, 1998; Brandstetter *et al.*, 1996). Differences in the amino acid sequence in these pockets between proteases may determine their affinity for specific substrates.

Summary

Vitamin K-dependent coagulation cascade serine proteases have extensive similarities in domain organisation and activation mechanism. However, differences in sequence confer unique structural features to each protease and govern their substrate specificity.

1.2.3 FIBRINOGEN

Structure of Fibrinogen

FGN is a 320 kDa glycoprotein secreted into the blood primarily by hepatocytes, where it reaches a concentration of approximately 9 μM with a half-life of around 100 hr (Herrick *et al.*, 1999, for review). The FGN monomer is formed of three pairs of non-identical chains; namely the Aα-, Bβ- and γ-chains (Henshen-Edman, 1995, for review). Diagram 5 (page 47) represents the structure of FGN.

The predominant Aα-chain of circulating FGN contains around 610 amino acids residues (70 kDa), the Bβ-chain of FGN consists of 461 amino acids (56 kDa) and the γ-chain consists of 411 residues (48 kDa). The alternative splicing which occurs in the Aα– and γ-chains and the heterogeneity of the extensive post-transitional modifications of the protein including phosphorylation, sulphation, glycosylation and hydroxylation lead to a the generation of a great variety of FGN monomers (Henshen-Edman, 1995, for review).

FBN which polymerase spontaneously into an insoluble mesh. Later, the FBN fibres are crosslinked by factor XIIIa, a transglutaminase which stabilises the FBN lattice (Shainoff *et al.*, 1991; Devine and Bishop, 1996) and gives it further resilience (Mosesson, 1998, for review). In addition, it has also been suggested that extravasated FGN may be deposited into the extracellular collagenous matrix without being processed by thrombin (Guadiz *et al.*, 1997). This FBN network fills the wound space and provides a haemostatic plug, in close association with aggregated and entrapped platelets, which prevents further blood loss.

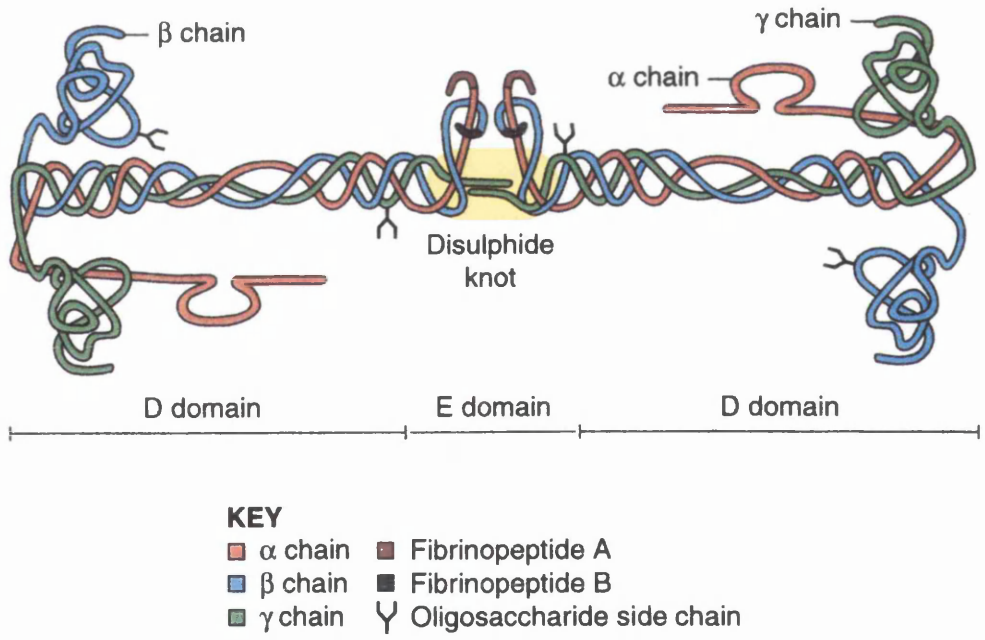


Diagram 5 **Schematic representation of the structure of fibrinogen**

In this diagram are represented the principal domains of the FGN molecule. The three types of chain pairs which constitute the backbone of the FGN monomer are represented in colour, the Aα-chains, the Bβ-chains and the γ-chains. The three pairs of FGN chains are held together by 29 disulphide bonds. The majority of these form a disulphide knot that ties together the amino terminal regions of all the chains. The fibrinopeptides cleaved by thrombin during blood coagulation are represented in darker colours.

FGN has three distinct domains delineated in part by the cleavage sites of plasmin: Two terminal D domains (67 kDa), each linked to a single central E domain (33 kDa) by a triple-stranded array of the polypeptide chains believed to exist in the form of α helical coiled coils. The three constitutive chains and the two halves of the FGN molecule are held together by a series of 29 disulphide bonds (Mosesson, 1998, for review).

Fibrin Deposition

The coagulation cascade is initiated at sites of vascular damage within seconds of blood protein extravasation. The coagulation proteases are activated in a series of stepwise reactions, resulting in the activation of prothrombin. Initially, active thrombin cleaves fibrinopeptide A off the amino terminus of each of the two FGN Aα–chains and subsequently fibrinopeptides B from the two Bβ–chains (Bilezikian *et al.,* 1975; Stubbs *et al.,* 1992). These reactions transform soluble blood FGN monomers into fibrin fibrils and a complex FBN network (FBN). These events will be discussed in details in section 5.1.1.

Fibrinolysis

As tissue repair progresses, FBN is cleared from the wound site and replaced by the mature scar tissue (Chung *et al.*, 1996, for review). At the site of trauma, inflammatory cells and fibroblasts (Schafer *et al.,* 1994) as well as platelets (Lenich *et al.,* 1997; Loscalzo *et al.,* 1995) release proteases such as urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) and tissue-type plasminogen activator (t-PA). Urokinase-type and tissue-type plasminogen activators catalyse the activation of plasminogen into plasmin and initiate the *fibrinolytic cascade.* One difference between these structurally related enzymes is that uPA is active on the surface of cells, whereas tPA gains its' activity following binding to the FBN E domain (Haddeland *et al.,* 1996; Angles-Cano, 1994, for review). Finally, plasmin cleaves FBN into a well characterised range of FBN degradation products (FDPs) distinct from those generated by thrombin (Walker and Niemshen, 1999). In this manner, FBN is cleared from the wound site and replaced by the forming and maturing scar tissue (Chung *et al.*, 1996, for review).

Plasminogen activation and fibrinolysis can be stimulated through the intrinsic coagulation pathway. The activation of the kallikrein-kinin system by factor XIIa on the surface of endothelial cells leads to the formation of kallikrein which in turn activates pro-urokinase-type plasminogen activator (pro-u-PA; Loza *et al.,* 1994), thereby initiating u-PA and plasminogen activation

(Lenich *et al.*, 1995). This suggests that the activation of blood coagulation is intrinsically linked to the activation of the fibrinolytic system.

Whereas plasmin activity provides the principal mechanism of FBN clearance from the wound site during healing (Walker and Nesheim, 1999), other proteases produced mainly by inflammatory cells can also degrade the FBN network. They include cathepsin D from macrophages (Simon *et al.*, 1994), mast cell tryptase (Schwartz *et al.*, 1985), neutrophil leukocyte elastase and cathepsin G (Kolev *et al.*, 1996; Adams *et al.*, 1995; Heidein *et al.*, 1996; for a review on inflammation proteases with fibrinolytic activity).

Summary

The activation of the coagulation cascade results in the localised deposition of FBN immediately after tissue injury. As healing progresses, the temporary FBN scaffold is degraded and replaced by a permanent collagen matrix. Thiese events give rise to a range of FGN degradation products generated through the action of thrombin during blood clotting, or other proteases during fibrinolysis, are present at the site of trauma during tissue repair.

1.3 SUMMARY AND AIMS OF THIS THESIS

1.3.1 SUMMARY

In this introduction, the biology of tissue repair and fibrosis has been reviewed with particular attention given to the generation of coagulation cascade proteases, protease cofactors and FGN cleavage products. This introduction has led to the following conclusions:

- Coagulation cascade peptides are activated at the time and location of trauma to prevent blood loss. Blood-borne coagulation factors activated via the extrinsic coagulation pathway are amongst the first mediators of homeostasis to be present at sites of injury.

- Following blood coagulation, collagen deposition is a central event of tissue repair. The production, deposition and remodelling of collagen is mainly mediated by fibroblasts. Thus, the appropriate regulation of fibroblast proliferation and collagen production at sites of tissue injury is essential to ensure normal tissue repair.

- Dysregulated fibroblast proliferation and excessive collagen deposition is a determinant factor in the pathogenesis of fibrotic diseases. In addition, there is persistent and excessive activation of the coagulation cascade at sites of tissue fibrosis.

- It is possible that thrombin and FGN cleavage may contribute to fibroblast proliferation and collagen deposition during normal tissue repair and fibrotic disorders. However, the effects of other coagulation cascade peptides generated during blood coagulation on fibroblast functions have not been determined. Indeed, it is unknown whether all coagulation factors or only thrombin are mitogenic for fibroblasts.

These gaps in our understanding of connective tissue deposition during tissue repair and fibrosis warrant further research on the role of coagulation factors on the regulation of fibroblast proliferation and procollagen production.

1.3.2 Overall Hypothesis

In the light of the preceding observations, the overall hypothesis of this thesis is that:

> Coagulation cascade peptides stimulate fibroblast proliferation

1.3.3 Overall Aims

This thesis has the following general goals:

- To assess the mitogenic potential of coagulation cascade factors VIIa, IXa, Xa, thrombin and FGN cleavage products that are generated via the extrinsic coagulation pathway on cultured fibroblasts. The potency and kinetics of these effects will be characterised in DNA synthesis and proliferation assays.

- To elucidate the molecular mechanism that transduces the mitogenic activity of coagulation cascade peptides using specific inhibitors, neutralising antibodies and fibroblasts derived from human tissues and genetically engineered animals.

1.3.4 STRUCTURE OF THIS THESIS

At the beginning of each result chapter (chapters 3, 4 and 5), the details of the literature necessary to understand the data will be summarised. Immediately afterwards, a specific hypothesis and detailed aims will be formulated for each chapter. Subsequently, the experimental results will be presented. The findings will be discussed in two ways:

First, the data obtained will be compared to the literature and their validity examined at the end of each chapter. Second, the implications of all the findings will be analysed together with respect to our understanding of protease biology, tissue repair and fibrosis in chapter 6.

CHAPTER 2

MATERIALS & METHODS

2.1 MATERIALS

2.1.1 COAGULATION AND GROWTH FACTORS

All chemicals were of analytical grade or above and obtained from BDH-Merck Ltd (Lutterworth, UK), unless otherwise indicated. All water used for the preparation of buffers was distilled and deionised using a Millipore Water Purification System (Millipore R010 followed by Milli-Q Plus; Millipore Ltd, Watford, UK). Solvents used for the preparation of high pressure liquid chromatography (HPLC) buffers and solutions were of HPLC grade and obtained from BDH-Merck.Ltd (Lutterworth, UK) Sterile tissue culture dishes, polypropylene centrifuge tubes and pipettes were obtained from Corning Costar Ltd (High Wycombe, UK), unless otherwise specified. Other disposable sterile plasticware was purchased from Sterilin Ltd (Ashford, UK). Sterile tissue culture medium, sterile tissue culture grade amino acids, trypsin/EDTA and antibiotics were obtained from Life Technologies Ltd (Paisley, UK). New born calf serum and foetal calf serum (heat inactivated) were purchased from Imperial Laboratories Ltd (Andover, UK).

Preparations of purified Russel viper venom-activated human factor Xa, factor IXa and factor VIIa and Dansyl-Glu-Gly-Arg Chloromethylketone dihydrochloride (DEGR-ck) were purchased from Calbiochem-Novabiochem UK Ltd (Nottingham, UK). For comparison purposes, a second commercial preparation of fXa was obtained from American Diagnostica Inc. (Greenwich, CT, USA). Proteolytically inactive DEGR-fXa was prepared, purified and provided by Dr C. Goodwin (National Heart and Lung Institute London, UK; See section on protease inhibition). Preparations of purified human thrombin, factor X and trypsin were purchased from Sigma Chemicals Ltd (Poole, UK). Human airway mast cell tryptase was purchased from Europa Bioproducts Ltd (Wicken, UK). All enzymes and inhibitors were prepared in DMEM culture medium, aliquoted and stored at -70°C. Recombinant human PDGF-AB was obtained from R&D Sytems Europe Ltd (Abingdon, UK) and was prepared in phosphate buffered saline supplemented with 0.1% bovine serum albumin, aliquoted and stored at -70°C.

2.1.2 PROTEASE INHIBITORS

Native hirudin, the specific thrombin inhibitor, was purchased from Sigma Chemicals Ltd (Poole, UK). Recombinant tick anticoagulant peptide (rTAP) was a kind gift from Dr M. Scully (National Heart and Lung Institute, London, UK) and originally prepared by Dr G.P. Vlasuk (Corvas International, San Diego, CA, USA). Antistasin core peptide was purchased from Bachem Ltd (Staffron Walden, UK).

TAP is a specific natural inhibitor of fXa isolated from the tick Ornithodoros moubata which abolishes fXa catalytic activity. TAP and its' recombinant equivalent rTAP bind to factor Xa in a reversible but highly selective fashion (Jordan *et al.,* 1992).

ASN is a specific natural inhibitor of fXa catalytic site isolated from the Mexican leech Haementeria offinalis. Short synthetic peptides mimicking the core inhibitory region of the molecule, including ASN D-Arg^{32}-Pro^{38} (ASN core peptide), have been shown to be active and abolish the catalytic activity of fXa selectively (Ohta *et al.,* 1994). Different size peptides derived from the sequence of antistasin were evaluated in cell function assays and ASN D-Arg^{32}-Pro^{38} represents the shortest region of antistasin with inhibitory activity.

Furthermore, these naturally occuring inhibitors present the advantage of being active in physiological fluids as well as highly selective for fXa. A variety of synthetic peptides mimicking TAP and ASN were used as the drugs of choice in several studies of antithrombotic agents. They were evaluated in various animal models and shown to have potent inhibiting properties at doses which do not induce bleeding complications (Wong *et al.,* 1996; Schwartz *et al.,* 1996; Ragosta *et al.,* 1994). Hence TAP and ASN provide a advantageous basis for the design of novel peptide inhibitors of fXa as potential antiproliferative and antifibrotic drugs.

2.1.3 ANTIBODIES

The polyclonal antibody (pAb) JC15 was a generous gift from Dr D. Altieri (Yale University, New Haven, CT, USA). The pAb JC15 is an anti-factor Xa antibody raised in mouse against a synthetic peptide mimicking the inter-EGF region Leu^{83}-Leu^{88} of human factor Xa (Altieri and Edgington, 1990). The pAb JC15 prevents the interaction between the protease and its' receptor EPR-1 in a competitive manner (Ambrosini *et al.,* 1997). The anti-EPR-1 mAb B6 was a generous gift from Dr D. Altieri (Yale University, New Haven, CT, USA). The mAb B6 was raised in mouse against a synthetic peptide mimicking the residues Pro^{120}-Ala^{154} of EPR-1 (Altieri and Edgington, 1990). The mAb B6 prevents the interaction between the protease and its'

receptor EPR-1 in a competitive manner (Ambrosini *et al.*, 1997). A monoclonal antibody 5224 (mAb 5224) directed against human factor Xa was supplied by American Diagnostica Inc. (Greenwich, CT, USA). The mAb 5224 is raised in mouse immunoglobulin G_1 against human factor Xa that recognises epitopes in the vicinity of the catalytic site and inhibits its' activity (Zacharski *et al.*, 1991; Ko *et al.*, 1996). The anti-PAR-1 mAb WEDE15 is a kind gift from Dr L. Brass (University of Pennsylvania, Philadelphia, PA, USA) and it is used for immunofluorescent labelling of the receptor on the surface of cells (Ramachadran *et al.*, 1997). The anti-rat PAR-1 pAb is a kind gift from Dr E. MacKie (University of Melbourne, Melbourne, Victoria, Australia). This antibody is raised in rabbit against a glutathione S-transferase-PAR-1 fusion protein and used for Western blot analysis. The pan specific anti-PDGF pAb was purchased form R&D Sytems Europe Ltd (Abingdon, UK). The anti-PDGF pAb immunoglobulin G_Z was raised in goat against natural human PDGF and neutralises the activity of all human isoforms of the growth factor (Herbert *et al.*, 1998).

2.1.4 HUMAN FOETAL, ADULT AND MOUSE PAR-1-DEFICIENT FIBROBLASTS

Human foetal lung fibroblasts (HFL-1), catalogue number CCL-153, were obtained from the American Type Culture Collection (Rockville, MD, USA) and were used at passages no higher than 20 without any noticeable modification of the tested parameters. These cells were selected for they are known to be highly responsive to thrombin and other coagulation cascade peptides in proliferation and procollagen production assays (Gray *et al.*, 1990; Gray *et al.*, 1995a; Chambers *et al.*, 1998). Furthermore, the more robust proliferative rate of these foetal cells makes them a useful model to study mitogenic agents. Novel findings made in human foetal lung fibroblasts were verified in primary human adult fibroblasts.

Primary cultures of Human adult fibroblasts were kind gifts from Dr M. Parsons (breast dermal fibroblasts), Dr L. Reynolds (left ventricular heart fibroblasts) and Dr C. Keerthisingam (lung alveolar fibroblasts) from University College London (London, UK). Primary fibroblasts were grown from 1 mm^3 explants dissected from normal human organs. The explants were cultured in culture medium supplemented with 10% serum. The culture medium was replaced with fresh medium one day after isolation and every three days thereafter for three weeks. Eventually, fibroblasts were collected by trypsinisation and characterised. All cells were characterised morphologically and by differential immunocytochemical staining for a selection of smooth muscle cell, endothelial and fibroblast markers such as α-smooth muscle actin, von Willebrand

factor, Vimentin and myosin. Human adult SV40-transformed kidney fibroblasts were a kind gift from Dr J. Norman (University College London, London, UK). The various primary cells, including SV40-transformed kidney fibroblasts, were discarded after 5 to 10 passages.

Wild type mouse lung fibroblasts and PAR-1-deficient mouse lung fibroblasts were a kind gift from Dr P. Andrade-Gordon, R.W. Johnson Pharmaceutical Research Institute (Spring House, PA, USA). These fibroblasts were immortalised through SV40 transformation and used between passage 20 and 35 without noticeable modification of tested parameters.

2.2 METHODS

2.2.1 TISSUE CULTURE CONDITIONS AND SUB-CULTIVATION

Cells were maintained in static culture on 75 cm^2 triangular culture flasks in Dulbecco's modified Eagle's medium (DMEM), supplemented with penicillin (200 units/ml), streptomycin (200 units/ml), glutamine (4 mM) and 10% (v/v) newborn calf serum (NCS) for fibroblasts and foetal calf serum (FCS). Plates were incubated at 37°C in a humidified atmosphere of air containing 10% CO_2. Cells were routinely tested for mycoplasma contamination using a probe for mycoplasma ribosomal RNA (Gen-Probe Mycoplasma T.C.II, Laboratory Impex Ltd, Teddington, UK).

Upon reaching visual confluence (6 to 7 days), cells were sub-cultured (passaged) into new culture flasks. The medium was removed and the cell layer was washed with 10 ml Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS) to remove any remaining culture media. Cells were brought into suspension by adding 1 ml of a trypsin (0.05%, w/v) / EDTA (0.02%, w/v) solution and incubating the cells at 37°C until cell detachment from the tissue culture plasticware occurred (approximately 2 min for fibroblasts). The cells were observed under an Olympus TCK-2inverted phase contrast light microscope (Olympus Optical Ltd, London, UK) to ensure that they had rounded up. Trypsin was neutralised by addition of 9 ml of either DMEM supplemented with 10% NCS. The cell suspensions were split at a ratio 1 to 6. Under these conditions, cells remained viable for about 10 to 20 passages after supply for human foetal lung fibroblasts, and up to 6 to 10 passages for primary human fibroblasts. Cells used for experiments were between passages 14 and 22 for human foetal and SV40-transformed fibroblasts, 3 and 10 for primary human fibroblasts.

To plate cells for experiments, cells from one tissue culture flask were put in suspension in DMEM-5% NCS and aspirated into a 50 ml sterile polypropylene centrifuge tube (Corning Costar Ltd, High Wycombe, UK). They were centrifuged (300 g, 7 min, 4°C) using a bench centrifuge

(MSE Mistral 3000, Loughborough, UK), prior to plating. The supernatant was discarded and the cell pellet was brought into a single cell suspension with 1 ml DMEM-5% NCS by gentle mixing. A further 9 ml culture media was added and an aliquot of the suspension was removed with a sterile pipette. Cells were counted using an improved Neubauer haemocytometer (BDH-Merck Ltd, Lutterworth, UK) and the density of the cell suspension was adjusted with DMEM-5% NCS.

2.2.2 CELL PROLIFERATION STUDIES

In this thesis, cell proliferation was assessed using three methods: The methylene blue dye binding assay, the ^{3}H-thymidine incorporation assay and all major results were confirmed by direct cell count.

For each individual experiment, a *negative control* composed of culture medium without additions (0% serum) and a *positive control* (10% serum) were systematically evaluated in addition to the various treatments investigated. In addition, several potent growth factors were also assayed for comparison, such as PDGF. The repeated evaluation of these controls allowed to control the normal behavior of the tested cells in each experiment and to further insure the reproducibility of the results between experiments.

2.2.2.1 Methylene Blue Proliferation Assay

An assay based on the uptake and release of methylene blue dye was employed to investigate the effect of coagulation cascade peptides on cell growth. Fibroblasts were plated in 96 well tissue culture plates in DMEM plus 5% NCS (5000 cells/ 100 µl/ well). In order to avoid edge effects, cells were only dispensed into the central 10 x 6 wells of the 96 well plate. The outer wells were filled with 100 µl DMEM-5% NCS only.

After 24 hr in culture and before confluence, the medium was removed and replaced with fresh DMEM supplemented with the test substances. All enzymes were evaluated in 0% serum, unless specified otherwise. After 48 hr in culture, cell replication was assayed using a spectrophotometric assay based on the uptake and subsequent elution of the dye methylene blue according to the method described by Oliver and colleagues (Oliver *et al.*, 1989). Cell proliferation experiments were performed at subconfluence, in the absence of serum.

Methylene blue is a base and possesses a positive charge at pH 8.5, and thus binds electrostatically to negatively charged groups, including nucleic acids with phosphate moieties and

negatively charged amino acids in proteins within the cell layer. The dye is eluted from the cell layer by lowering the pH to below 2 with hydrochloric acid. This results in the protonation of acidic groups and liberation of methylene blue into the eluent. Methylene blue forms dimers in aqueous solution at low pH and does not obey Beer-Lambert's Law. This is suppressed by using a hydrochloric acid/ethanol eluent, resulting in a single absorbance peak at 650 nm.

2.2.2.2 Direct Cell Counting

For direct cell count experiments, cells were plated in 24 well tissue culture plates in DMEM plus 5% NCS (60000 cells/ 2.5 ml/ well). After 24 hr in culture and before confluence, the medium was removed and replaced with fresh DMEM supplemented with the test substances. All enzymes were evaluated in 0% serum, unless specified otherwise.

After 48 hr in culture, adherent cells were detached with trypsin / EDTA (0.05-0.02% respectively). Cell number was determined by counting in a hemacytometer. Four to eight replicate wells were counted for each determination.

2.2.2.3 DNA Synthesis Assay

Cells were plated in 5% NCS for 24 h in a similar fashion to the proliferation assay, quiesced in 0% NCS for 24 hr and used at subconfluence. The medium was removed and replaced with fresh DMEM supplemented with the test substances. All enzymes were evaluated in 0% NCS. After 16 hr in culture, ^{3}H-thymidine (2 μCi/ml) was added.

The schedule of incubation with the radioactive label was optimised to obtain the greatest stimulation over controls. For this purpose, a 4 hr *'pulse labelling'* was used at the time corresponding to the greatest DNA synthetic activity in stimulated fibroblasts, about 18 hr after the mitogenic stimulus (figure 2.1).

Although less sensitive, a second labelling schedule was used to detect the effect of slow acting mitogens. For this purpose the cells were labelled from 16 hr to 48 hr of incubation. This method was termed *'continuous labelling'* (figure 2.1). The peak of DNA synthesis following treatment with serum of the fibroblasts occurs at about 18 hr after stimulation. Over 75% of the total radioactive label incorporation allowed by *continuous labelling* (16 hr to 48 hr after stimulation) occurs over 4 hr of *pulse labelling* (16 to 20 hr after stimulation, figure 2.1).

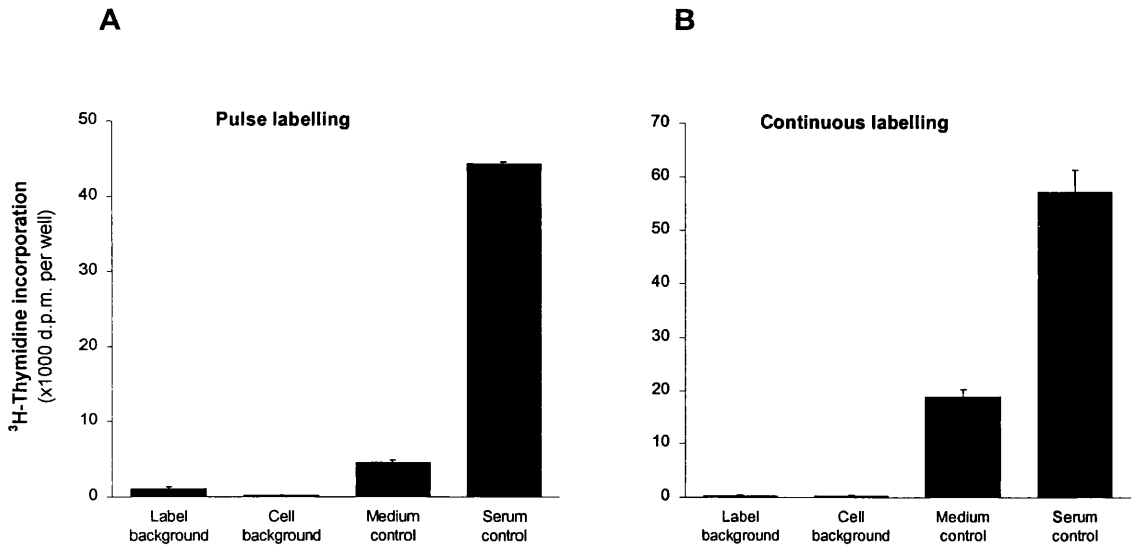


Figure 2.1 ^{3}H-thymidine incorporation assay: Pulse and continuous labelling methods of *de novo* DNA synthesis labelling

This figure shows the ^{3}H-thymidine labelling levels into growth arrested (Control) and serum-stimulated fibroblasts (10% NCS), as well as radioactivity levels measured after non-specific binding of the radiolabel to tissue culture plastic and basal radioactivity levels detected in non-labelled cells. The addition of latter two values gave a global background value which was subtracted from all other data.

A 4 hr **Pulse labelling** (16 to 20 hr after stimulation);

B 32 hr **Continuous labelling** (16 to 48 hr after stimulation).

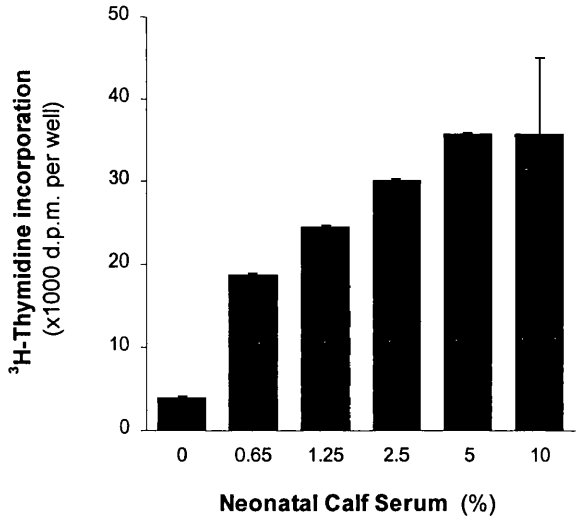


Figure 2.2 Effect of serum on fibroblast DNA synthesis

This figure shows the effect of NCS on growth arrested fibroblast DNA synthesis, as measured by ^{3}H-thymidine incorporation following the pulse labelling method. NCS stimulated fibroblast DNA synthesis significantly and in a dose-dependent fashion from 0.65% to 10%. The cells were maximally stimulated from 5% NCS onwards ($p<0.01$) and higher percentages of serum did not stimulate extra DNA synthesis significantly. In contrast, low concentrations of serum, such as 0.65% NCS stimulated an increase of DNA synthesis of over 300% above medium control ($p<0.01$). This graph demonstrates the sensitivity of the assay, in particular for low concentrations of mitogens, and the amplitude of increase stimulated by higher concentrations of mitogens.

After radiolabelling, the cells were then frozen and thawed once before harvest. Subsequently, the DNA was harvested on Titertek filterpaper from ICN Inc (Costa Meas, CA, USA) with a *micro96* cell harvester from Skatron (UK). Filters were washed twice with an excess amount of H_2O and cut in pieces for each well that were shaken in 5 ml of scintillation fluid Ecoscint A (National Diagnostics Inc., Atlanta, GA, USA) in Pico 2000 polyethylene vials from Packard Instruments Inc (Meriden, CT, USA). The samples were left to rest in the dark overnight.

For the measure of incorporated radioactivity, the samples were processed through a Miniaxiβ liquid scintillation counter from Packard Instruments Inc (Meriden, CT, USA). The background values due to unlabelled cells in culture or the non-specific adsorbtion of ^{3}H-thymidine onto plastic were subtracted from all experimental values.

The final data was measured in disintegrations per minute (d.p.m.) and expressed in percent stimulation above control values. NCS is a well known and potent mitogen and it stimulated fibroblast proliferation in a dose-dependent manner (figure 2.2). The effect of serum (10% NCS) on fibroblast DNA synthesis was used as a positive control in each experiment.

2.2.2.4 Chelation of Cytosolic Free Calcium

To assess the role of free cytosolic calcium mobilisation in protease-stimulated fibroblast proliferation, cells were depleted from free cytosolic calcium by loading with the highly specific Ca^{2+} chelator 1,2-bis(o-aminophenoxy)-ethane-N,N,N',N',-tetra-acetic acid tetra(acetoxymethyl-ester) or BAPTA-AM (Molecular Probes Europe BV, Leiden, The Netherlands). The molecule is derivatised as an acetoxymethyl-ester to make it cell permeant. The acetoxymethyl (AM) tail allows the compound to cross cell membranes and reach the cytosol. Once in the intracellular compartment, cytosolic esterases remove the acetoxymethyl-ester tails off the molecule, leaving the chelator able to interact and chelate with free calcium ions with great specificity over other divalent cations (Tsien, 1980). BAPTA is commonly used to deplete mesenchymal cells from cytosolic free calcium and abrogate cellular responses to proteases in vascular smooth muscle cells (Cizmeci-Smith *et al.*, 1997) or in fibroblasts (Ha and Exton, 1993).

Cells were plated in 5% NCS for 24 hr in a similar fashion to proliferation assays and used at subconfluence. The medium was removed and the cells were washed once with fresh DMEM (no serum). This had the purpose of removing traces of esterase activity present in the culture medium since esterase processing of BAPTA-AM outside the cells would compromise loading.

The cells were then loaded with BAPTA-AM by incubation for 2 hr with the chelator prepared in DMEM. BAPTA-AM was initially dissolved and stored at -70°C and aliquoted in

dimethyl sulphoxide. The cells were then washed again in DMEM (without additions) to remove excess or extracellular BAPTA-AM. Loaded cells were then treated with the relevant treatments in the absence of serum and assayed for DNA synthesis after 20 hr or proliferation after 48 hr.

2.2.3 PROTEIN STUDIES

2.2.3.1 Total Protein Synthesis Assay

Cells were plated in 5% NCS for 24 hr in 24 well tissue culture plates, quiesced in 0% NCS for 24 hr and used at subconfluence. At the start of the experiment, the medium was removed and replaced with fresh DMEM supplemented with test substances. All enzymes were evaluated in the absence of serum.

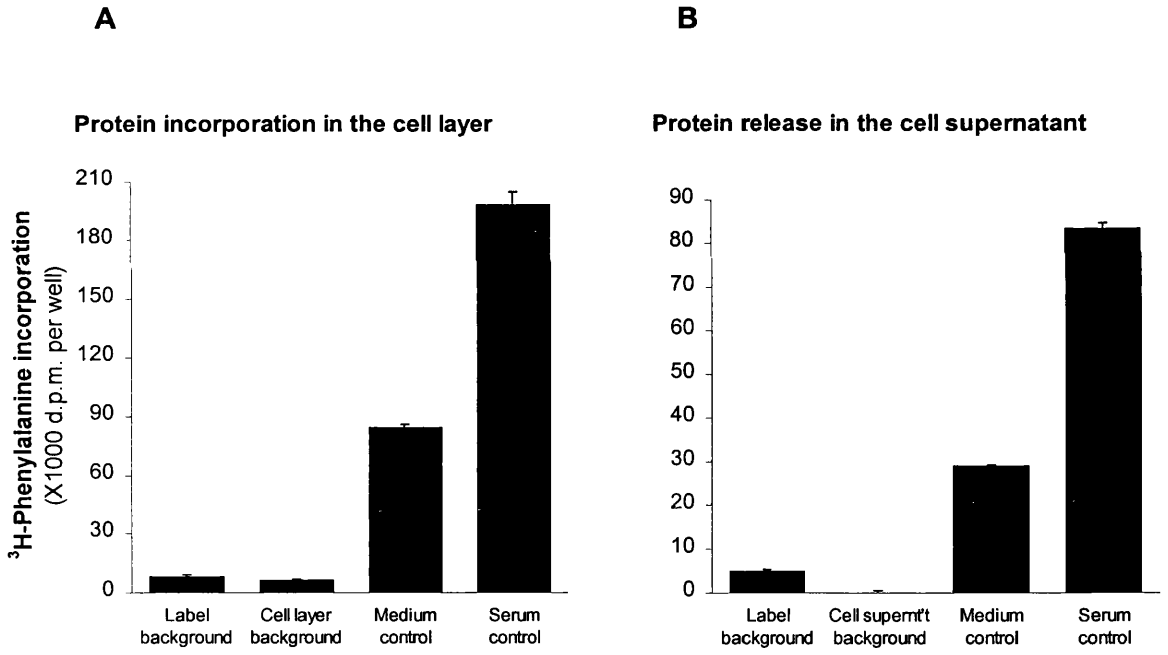


Figure 2.3 ^{3}H-phenyalanine incorporation assay: *De novo* protein synthesis labelling methods in cultured cell layers and supernatants

This Figure shows the ^{3}H-phenylalanine labelling levels into growth arrested (Control) and serum-stimulated fibroblasts (10% NCS), as well as radioactivity levels measured after non-specific binding of the radiolabel to tissue culture plastic and basal radioactivity levels detected in non-labelled cells. The addition of latter two values gave a global background value which was subtracted from all other data.

A Incorporation into the **cell layer** (Protein deposited);

B Incorporation into the **cell supernatant** (Protein secreted).

^{3}H-phenylalanine (2 μCi/well) was added to the cells simultaneously to the treatments for the labelling of *de novo* protein synthesis. The cells were incubated for 48 hr. At the time of harvest, the cell supernatants were collected and transferred to polypropylene tubes. The cell culture supernatant proteins and those of the cell layer were collected, processed and analysed separately to allow the distinction between newly synthesised proteins deposited into the cell layer substratum and those secreted into culture medium.

Cell supernatant proteins were precipitated by mixing with an equal volume of ice cold trichloroacetic acid (10%), followed by vigorous shaking and overnight incubation at 4°C. The protein was subsequently collected into a pellet at the bottom of the tubes by centrifugation at 15000 *g* for 10 min with a CS15r centrifuge (Beckman Ltd, High Wycombe, UK). The supernatant was removed and the pellet was hydrolysed in 250 μl of sodium hydroxyde for 24 hr at room temperature. The tissue culture wells were rinsed very carefully in ice cold PBS so as not to disturb the cell layer and the cell layers were frozen and thawed once. The contents of each well were hydrolysed in 250 μl of sodium hydroxyde for 24 hr at room temperature with agitation.

The radioactively labelled hydrolysates from the cell layers and those of the cell supernatant proteins were then added to 5 ml of scintillation fluid Ecoscint A from National Diagnostics Inc (Atlanta, GA, USA) in Pico 2000 polyethylene vials from Packard Instruments Inc (Meriden, CT, USA). The samples were left to rest in the dark overnight.

Finally, the incorporated radioactivity was measured in a Miniaxiβ liquid scintillation counter from Packard Instruments Inc (Meriden, CT, USA). The background values due to unlabelled proteins or the non-specific binding of ^{3}H-phenylalanine to plastic were subtracted from all experimental values (figure 2.3, panels A and B). The final data was measured in disintegrations per min (d.p.m.) and expressed in percent stimulation above control values.

2.2.3.2 Procollagen Synthesis Assay

Procollagen production by cultured fibroblasts was assessed by measuring hydroxyproline (Hyp) in intact proteins (ethanol-insoluble fraction) using a high-pressure liquid chromatography method previously developed in this laboratory (Campa *et al*, 1990; McAnulty *et al*, 1991).

For each individual experiment, a negative control composed of culture medium without additions (0% serum) and a positive control (TGF-β1) were systematically evaluated in addition to the various treatments investigated. In addition, several potent growth factors were also assayed for comparison, such as PDGF.

Hyp represents approximately 12% of the primary sequence of procollagen and is essential for the formation of the collagen triple helix. This amino acid however, is not present in a significant level in most proteins. The ones which have been reported to contain Hyp are elastin, complement protein C1q, acetylcholinesterase, surfactant apolipoproteins A and D, ficollins and macrophage scavenger protein. Fibroblasts in culture have not been shown to produce these proteins, therefore Hyp levels measured in fibroblasts *in vitro* is an excellent indicator of procollagen concentrations.

Cell Culture Conditions

Cells from confluent cultures were brought into suspension as described previously and seeded at a cell density of 30,000 cells/well in 0.5 ml DMEM-5%NCS into 24 well sterile culture dishes. Upon reaching confluence (after 5-6 days), the culture medium was removed and replaced with 0.5 ml serum-free DMEM, supplemented with freshly added glutamine (4 mM), penicillin (200 units/ml), streptomycin (200 units/ml), ascorbic acid (50 μg/ml), proline (0.2 mM), (pre-incubation media), without disrupting the fragile cell monolayer. After 24 hr, the medium was replaced by incubation medium, consisting of DMEM supplemented as described above. Cells were returned to the incubator for up to 48 hr (n = 6 for each treatment).

Since there is a small amount of procollagen in the cell layer, samples consisting of freshly added incubation medium into which the cell layer has been scraped were also taken at the onset of the incubation period (t_0), for each experiment.

Cell Harvesting

At the end of the incubation period, the cell layer was scraped into the culture medium with a teflon scraper and the contents of each culture dish/well were aspirated and transferred to glass tubes. Culture wells were washed with cold phosphate buffered saline (PBS) which in turn was combined with the initial aspirate. All samples were frozen immediately at -40°C to avoid proteolysis.

Separation of Ethanol-Insoluble and Ethanol-Soluble Fractions

Samples were eventually thawed on ice and proteins were precipitated by addition of absolute ethanol to a final concentration of 67% (v/v) at 4°C and overnight (16 hr). Ethanol-insoluble proteins (precipitated proteins) were separated from ethanol-soluble moieties (amino acids and small peptides) using filtration through an acid resistant 0.45 μm pore filter (type HV, Millipore Ltd, Watford, UK) using a vacuum filtration unit (Millipore Ltd, Watford, UK). Filters with adherent proteins were transferred to hydrolysis tubes and the filters were resuspended and hydrolysed in 3 ml

HCL (6 M) at 110°C for 16 hr. Hydrolysates were decolourised with 30 mg of activated charcoal (Hopkins and Williams, Essex, UK) and filtered through an acid resistant 0.65 μm pore membrane (type DA, Millipore Ld, Watford, UK).

Principle of Measurement of Hydroxyproline by Reverse-Phase High Pressure Liquid Chromatography

Hyp in sample hydrolysates was isolated and measured by reverse-phase-HPLC following derivatisation with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl; Sigma Chemicals Ltd, Poole, UK) according to the original method described by Campa *et al.* (1990). Under the conditions described below, secondary amino acids react with NBD-Cl to produce a chromophore exhibiting a strong light absorbance at 495 nm. NBD-Cl also reacts with primary amino acids but these amino acids only have limited absorbance at this wavelength. In addition, the reaction between Hyp and proline occurs one order of magnitude faster for secondary amino acids (Ahnoff *et al.*, 1981). Interference from primary amino acids is therefore minimised by keeping the derivatisation time to 20 min, a time point at which the extent of Hyp derivatisation with NBD-Cl at 37°C was previously shown to be maximal for up to 20 nmol Hyp (Campa *et al.*, 1990).

Pre-Column Derivatisation

A 150 μl aliquot of each sample hydrolysate, prepared as described above, was transferred to a microfuge tube and evaporated to dryness under vacuum on a sample concentrator (Savant Speedvac Plus AR SC110 AR from Life Science International, Basingstoke, UK). In order to avoid contamination during the drying procedure, the mouth of the tube was covered with perforated parafilm. The residue obtained was redissolved in 100 μl of HPLC grade water, buffered with 100 μl of 0.4 M potassium tetraborate (pH 9.5), (Sigma Chemicals Ltd, Poole, UK) and reacted with 100 μl of 36 mM NBD-Cl in methanol to a final concentration of 12 mM NBD-Cl. NBD-Cl-derivatised amino acids are light sensitive, so the samples were protected from light by wrapping the microfuge tube in aluminium foil. The samples were incubated in a water bath at 37°C for 20 min, after which time the reaction was stopped by lowering the pH with 50 μl of 1.5 M hydrochloric acid. After adding 150 μl of a concentrated solution of HPLC running buffer A (167 mM sodium acetate in 26 % aqueous acetonitrile, pH 6.4), samples were filtered using an HPLC low dead volume filter (pore size 0.22 μm, type GV; Millipore Ltd, Watford, UK). Finally, a 100 μl aliquot was injected onto the HPLC column and eluted with an acetonitrile gradient as described below.

Instrumentation and Chromatography Conditions

Derivatised samples were separated on a Beckman System Gold HPLC system (Beckman Ltd, High Wycombe, UK), with a reverse-phase cartridge column (LiChroCART-LiChrospher ,250 mm length x 4 mm diameter, 5 μm particle size, 100 RP-18; BDH-Merck Ltd, Lutterworth, UK) protected by a directly coupled precolumn (LiChrosorb, 4 mm length x 4 mm diameter, 5 μm particle size, 100 RP-18; BDH-Merck Ltd, Lutterworth, UK). The columns were continuously maintained at 40°C in a heated oven. At the beginning of each experiment, the running buffers were degassed with helium (BOC Ltd, London, UK) and the HPLC system equilibrated in running buffer A for 40 min. The first two samples derivatised were Hyp standard solutions (equivalent to 50 and 100 pmol) which were then used for calibration.

NBD-Cl derivatives in samples and standards were eluted with an acetonitrile gradient, generated by gradually changing the relative proportions of the running buffers, thereby increasing the concentration of acetonitrile over time. The chromatographic conditions employed are summarised in table 1.

Column	**LiChrosopher, 100 RP-18, 250 mm length, 4 mm diameter, 5 μm pore**
Mobile phase	A - aqueous acetonitrile (8% , v/v) 50 mM Sodium acetate, pH 6.4 B - aqueous acetonitrile (75%, v/v)
Column flow rate	1.00 ml/min
Column temperature	40°C
Wavelength for detection	495 nm
Buffer mix **Elution gradient : (about 12 min)** **Purge column from buffer B :**	Time (min) / Proportion of buffer B (%) 0 / 0 5 / 5 12 / 80 12.5 / 0 25 / 0

Table 1 Conditions and buffers for the separation of hydroxyproline by HPLC

Post-column detection was achieved by monitoring absorbance at 495 nm using a flow-through variable wavelength monitor. The signal was processed on an on-line chromatography computing integrator (System Gold, Beckman Ltd, High Wycombe, UK) for quantitation.

Hyp elutes from the column between 5 and 7 min after injection, between glutamine (3.5 min) and serine (7 to 9 min) and just prior to the mobile phase becoming predominantly organic. Remaining amino acid derivatives in the sample were eluted as the hydrophobicity of the acetonitrile organic buffer was increased. Hyp in ethanol-insoluble proteins separated from other moieties as a well defined peak. The column running and regeneration time was 25 min.

Quantitation of Hydroxyproline

Quantitation of the Hyp content in each 150 µl sample injected onto the column was determined by comparing peak areas of chromatograms obtained for each sample to those generated from the two standard solutions derivatised and separated under identical conditions at the beginning of each experiment. All values were corrected for the amount of Hyp in the ethanol-insoluble fraction of the t_0 sample, representing protein-bound hydroxyproline measured in the cell layer and culture medium at the onset of the incubation period.

Hyp measured in the ethanol-insoluble fraction (after subtraction of the t_0 value) was taken to represent procollagen produced by the cells over the incubation period. Data is expressed as procollagen production in nmol of Hyp per 48 hr per well.

2.2.3.3 Western Blot Protein Analysis

Cell Culture Conditions and Sample Collection

HFL1 fibroblasts were grown in 12 well tissue culture plates in the presence of DMEM and 5% NCS. The cells were grown to about 75% confluence. At the start of the experiment, the medium was then removed and replaced with fresh medium (0% NCS), with or without mediators for a selected length of time. Each result section will contain the particular experimental details. After the treatment period, cells were washed twice with ice cold PBS. Cells were kept on ice and lysed in 100 µl of Laemmli sample buffer (10% glycerol, 62.5 mM Tris-HCl, 2% SDS w/v, 0.02% w/v bromophenol blue, pH 6.8 with 5% 2-β-mercaptoethanol; Maniatis *et al.,* 1989) supplemented with a complete protease inhibitor cocktail ('Complete-Mini' pellets from Boehringer Mannheim, Lewes, UK). Whole cell extracts were stored in 20 µl aliquots at -70°C.

Electrophoresis

For electrophoresis, 20 µl aliquots of whole cell protein extract were boiled for 5 min immediately prior to loading onto an acrylamide gel. Each sample was briefly spun down on a bench-top centrifuge and loaded into a 7% acrylamide stacking gel with 12% acrylamide separating gel in an XCell II electrophoresis tank (Novex Inc./R&D Sytems Europe Ltd, Abingdon, UK). A pre-stained 4 kDa to 250 kDa apparent molecular mass (M_r) ladder (SeeBlue^{TM}; Novex Inc./R&D Sytems Europe Ltd, Abingdon, UK) was run in a separate lane to easily identify various M_r. Samples were electrophoresed at 125 V for 3 hr in running buffer (Maniatis *et al.*, 1989).

The proteins separated in the experimental gel were then transferred onto a Hybond-ECL filter (Amersham Pharmacia Biotech Ltd, St Albans, UK) for 1 hr at 25 V. The filter was then rinsed once in double distilled water. To assess equal transfer, the filter was briefly stained in 2% Ponceau red solution (Sigma Chemicals Ltd, Poole, UK) and de-stained by a quick wash in double distilled water followed by a wash in PBS containing 0.1% Tween20 (PBST). Filters were wrapped in cling film for storage at -70°C before enhanced chemiluminescence detection.

Enhanced Chemiluminescence Protein Detection

Protein expression was investigated by using specific antibodies, including mAb B6 raised in mouse against EPR-1 (1:100 dilution) and an antibody raised in rabbit against PAR-1 (1:1000 dilution; See section 1.2 for details on antibodies). The primary and secondary antibodies were prepared in PBST containing 2% milk and 2% BSA.

Western blot filters were blocked using PBST as well as 2% milk and 2% BSA for 30 min at room temperature with gentle agitation. Filters were then incubated overnight at 4°C with the primary antibody and rinsed once with PBST only for 5 min. Subsequently, the specific primary antibodies were detected with anti-mouse and anti-goat (1:2000 dilution) horseradish peroxidase-conjugated antibodies (Dako Ltd, High Wycombe, UK). At the end of this incubation period, filters were washed three times 5 min with PBST only. Excess wash solution drained off.

Subsequently, 1 ml to 2 ml of enhanced chemiluminescence detection reagent (Amersham Pharmacia Biotech Ltd, St Albans, UK) was then spread over the filters and incubated for 1 min after what excess reagent was drained off. Finally, filters were wrapped in cling film and immediately exposed to autoradiography film (Kodak X-omat; Sigma Chemicals Ltd, Poole, UK) for 10 sec (PAR-1 detection) to 20 min (EPR-1 detection).

2.2.4 mRNA STUDIES

2.2.4.1 Preparation of the EPR-1 cDNA Probe

An EPR-1 cDNA probe was prepared from the full length EPR-1 cDNA construct kindly provided by Dr D. Altieri (Yale University, New Haven, CT, USA). The 1.6 kb EPR-1 encoding sequence was cloned into an EcoRI cloning site in a 2.96 kb pBluescript II KS (+/-) vector (Stratagene Europe Ltd, Cambridge, UK) containing an ampicillin-resistance gene.

Bacterial Transformation

A 50 µl aliquot of Escherichia coli cells, DH5α-*Subcloning Efficiency* strain (Life Technologies Ltd, Paisley, UK) in 50% glycerol and 50% LB broth was thawed on ice. The bacteria were then incubated with 5 µg (1µl) of the EPR-1 cDNA construct in TE buffer (Tris-Cl 10 mM, EDTA 1mM, pH 8.0) for 30 min on ice. For transformation, the cells were heat-shocked for 45 sec at 42°C in a waterbath and immediately put on ice for 2 min. Subsequently, 450 µl of SOC buffer (bacto-tryptone 20 g/l, bacto-yeast extract 5 g/l, NaCl 0.5 g/l, KCl 2.5 mM, pH 7.0, sterile; Before use add 10 mM $MgCl_2$ and 20 mM glucose) were added to the cells which were then shaken for 1 hr at 37°C at 220 rpm.

Bacterial culture

The cells were then streaked onto LB-agar plates (30 ml of LB-agar mix from BDH-Merck Ltd, Lutterworth, UK, poured into 10 cm diameter Petri dishes) containing ampicillin (50 µg/ml; Sigma Chemicals, Poole, UK). The plates were then incubated overnight at 37°C. Two ampicillin resistant colonies were then picked off the plates and seeded into 2 ml of LB broth (BDH-Merck Ltd, Lutterworth, UK) containing ampicillin in 14 ml snap-cap tubes (Vials 2059, Falcon Inc, Marathon Lab Supplies, London, UK) and grown over one day at 37°C in an orbital shaking-incubator. At the end of the day, the 2 ml cultures were inoculated into 250 ml of LB broth containing ampicillin (50 µg/ml) in a 1 L conical flask and grown overnight in LB broth at 37°C in an orbital shaking-incubator at 220 rpm.

Plasmid Extraction

The plasmid was prepared from bacteria with a Qiagen Maxiprep kit following the manufacturer's protocol (Qiagen Ltd, Crawley, UK). Briefly, the bacterial cells were lysed with a

NaOH-SDS based buffer. Proteins and plasmid DNA were precipitated high salt and later adsorbed on a DNA-binding column and eluted with TE buffer. The concentration of plasmid in the final preparation was determined by spectrophotometry at 260 nm (1 optical density unit at 260 nm = 50 μg/ml of DNA). 10 μg of plasmid was then digested with 10 units of EcoRI for one hr at 37°C in buffer H (all from Promega Life Science Ltd, Southampton, UK).

EPR-1 Construct Preparation

The digestion products (pBluescirpt® II KS vector and EPR-1 insert) were then separated by electrophoresis in 1% low melting point agarose gel (45 V for 4 hr). The 1.6 kb EPR-1-encoding insert was then cut out of the gel with a sterile scalpel. Each piece of gel containing the EPR-1 insert was melted in three times its' volume of double distilled water in a pre-weighed eppendorf tube. After analysis by spectrophotometry of the DNA concentration, it was adjusted to 25 ng/15μl. The EPR-1 insert was then stored at -70°C.

2.2.4.2 cDNA Probes

The PAR-1 probe is a fragment encompassing the coding sequences (CDS) from the human cDNA nucleotide 1 to 2123 Genbank accession number M62424 (Vu *et al.*, 1991). The PAR-2 probe is a 432 bp polymerase chain reaction product derived from the murine PAR-2 CDS, nucleotides 226 to 657 Z48043 (Nystedt *et al.*, 1996; Santulli *et al.*, 1995). The PAR-3 probe is a polymerase chain reaction product containing the human PAR-3 CDS nucleotides 145 to 1364 Genbank accession number U92971 (Ishihara *et al.*, 1997). The PAR-4 probe is a partial human CDS cDNA fragment, nucleotides 329 to 786, Genbank accession number AF055917 (Xu *et al.*, 1998). The β-actin probe is a human cDNA fragment from Clontech Ltd (Basingstoke, UK) and used for normalisation.

The EPR-1 probe is a 1.6 kb cDNA fragment encompassing the full length of the EPR-1a mRNA splice variant (Ambrosini *et al.*, 1997; section 2.2.4.1 for details).

2.2.4.3 Northern Blot Analysis

Precautions Taken to Prevent RNase Activity

For all experiments involving RNA isolation, deionised water was pre-treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) overnight at 37°C. The treated water was then autoclaved to

cause the breakdown and inactivation of the DEPC. All subsequent reagents were made from molecular biology grade chemicals (Sigma Chemicals Ltd, Poole, UK, unless otherwise stated) with DEPC-treated deionised water.

mRNA Isolation and Quantitation

Cells in culture were incubated with the treatments indicated for each experiment and prepared in standard cell culture medium for various incubation periods. The cells were plated in 60 mm diameter cell culture dishes (Corning Costar Ltd, High Wycombe, UK). At the end of each incubation period, the culture medium was removed and the cell layer washed with sterile PBS. The cell layer was then scraped into 1 ml of TRIzol reagent (Life Technologies Ltd, Paisley, UK) and the mRNA isolated according to the manufacturers instructions. The homogenate was incubated at room temperature for 10 min, followed by the addition of 200 µl of chloroform and centrifugation at 12,000 *g* for 15 min at 4°C. The aqueous phase was aspirated into a new tube and 500 µl of isopropanol were added to each sample. Following 10 min of incubation at room temperature, the samples were centrifuged at 12,000 *g* for 10 min at 4°C. Supernatants were then discarded and the pellets washed with 1 ml of 75% ethanol. Samples were centrifuged at 7,500 *g* for 5 min at 4°C and the supernatants were removed. Pellets were air-dried (although not completely) and resuspended in 50 µl of DEPC-treated water. RNA samples were stored at -70°C.

For RNA quantification, 2 µl of each RNA sample was diluted in 1 ml of water. The absorbance of each sample was sequentially measured at a wavelength of 260 nm and 280 nm using a spectrophotometer (Gilford 2000, Gilford Instrumentation Laboratories, Oberlin, Ohio, USA). A ratio optical density units at 260 nm/ optical density units at 280 nmwas used as a gross indicator of the purity of the RNA sample. Ratios between 1.6 and 1.9 are considered acceptable.

Calculations of RNA concentrations were based on the assumption that 40 µg of total RNA in 1 ml gives an absorbance reading of 1 optical density unit at 260 nm. This volume of RNA was then mixed with double the volume of RNA loading buffer containing ethidium bromide and heated to 65°C for 10 min, prior to loading into an agarose gel.

Poly-A^+ mRNA Isolation

Total messenger RNA (mRNA) was isolated from CHFR-298 by Dr A. Darrow at R.W. Johnson-Pharmaceutical Research Institute (Spring House, PA, USA). Total poly-A^+ mRNA was extracted electromagnetically from total RNA using biotinylated oligo-dT and streptavidin MagneSphere paramagnetic particles (Promega Life Science Ltd, Southampton, UK). One to 3 µg

of poly-A^+ mRNA were then mixed with loading buffer and loaded into gels for electrophoresis. This mRNA was used as a positive control for the detection of PAR-3 and PAR-4 mRNA. Alternatively, mRNA from human foetal lung fibroblasts was isolated using the Oligotex mRNA isolation kit according to the manufacturer's instructions (Quiagen Ltd, Crawley, UK).

Electrophoresis and Transfer to Nylon Hybridisation Filters

To prepare a 1% agarose gel, 2 g of agarose were added to 162 ml of DEPC-treated water and heated up in a microwave. After cooling the agarose solution down to 65°C, formaldehyde to 9% (v/v; about 18 ml) and 20X MOPS (0.4 M 3-N-morpholino-propane sulphonic acid, 32 mM sodium acetate and 4 mM EDTA; about 20 ml) were added. The gel was then cast into a gel tray and left to set at 4°C for 30 min. The gel was finally submerged in running buffer (1X MOPS).

The RNA samples were loaded into wells of the agarose gel and separated by electrophoresis in a general purpose electrophoresis box (International Biotechnologies Inc., CT, USA) at a constant voltage of 80 V in 1X MOPS running buffer for 2 hr to 3 hr. The integrity of the RNA and uniformity of the quantity loaded from each RNA sample was confirmed by the presence of the 18S and 28S ribosomal RNA species observed as prominent bands stained with ethidium bromide. The gel was photographed under UV light to visualise the ethidium bromide and subsequently washed in DEPC-treated water for 20 min. The RNA was transferred to a 'Hybond N' nylon filter (Amercham Pharmacia Biotech Ltd, St Albans, UK) prewetted with running buffer by overnight Northern blotting in 20X SSC (sodium chloride/sodium citrate solution: 175.3 g/l NaCl, 88.4 g/l sodium citrate) and following established protocols (Maniatis *et al.*, 1989). The RNA transferred to the filter was cross-linked to the filter using a Stratalink ultraviolet cross-linker (Stratagene Europe Ltd, Cambridge, UK).

Hybridisation and Autoradiography

Nylon filters were pre-hybridised in 5X Denhardt's solution (10 g/l Ficoll, 10 g/l polyvinylpyrolidone and 10 g/l bovine serum albumin), 5X SSC, 0.1% SDS (sodium dodecyl sulphate) and 100 µg/ml denatured salmon sperm DNA (Life Technologies Ltd, Paisley, UK) for at least 1 hr at 65°C in rotating hybridisation oven (Bachofer, Germany). During this time, the cDNA probes (section 2.1) were radiolabelled with ^{32}P-dCTP using the Megaprime random priming DNA labelling kit (all from Amersham Pharmacia Biotech Ltd, St Albans, UK) according to the manufacturers instructions. Pre-hybridisation solution was then discarded and replaced with

hybridisation solution (identical to pre-hybridisation solution, minus salmon sperm) and the radiolabelled probe was also added to the filter.

The detection of PAR-1, PAR-2, PAR-3 and PAR-4 mRNAs was done in collaboration with Dr A. Darrow (R.W. Johnson-Pharmaceutical Research Institute, Spring House, PA, USA). For the detection of these receptors' mRNAs, the hybridisation with the labelled cDNA probe was allowed to occur at 62°C for approximately 3 hr using Rapid-hyb buffer (Amersham Pharmacia Biotech Ltd, St Albans, UK). After hybridisation, filters were washed at 62°C, twice with 2X SSC and 0.1% SDS and twice with 0.2X SSC 0.1% SDS.

For the detection of EPR-1, labelled cDNA probe was added to the hybridisation solution and the filter incubated overnight at 65°C. After hybridisation with the EPR-1 cDNA probe, filters were washed once in 2X SSC, 1% SDS for 10 min at room temperature by hand, once in 2X SSC, 0.5% SDS for 10 min at room temperature by hand and once in 1X SSC, 0.1% SDS for 10 min at 65°C in shaker incubator (high stringency wash).

Filters were then wrapped in cling film and exposed to autoradiographic film (Sigma Chemicals Ltd, Poole, UK) at -70°C with intensifying screens until a suitable signal was visible: 18 hr of exposure time (ET) for PAR-1; 8 days ET for PAR-2; 5 days ET for PAR-3; 5 days ET for PAR-4; 18 hr ET for β-actin and 5 days ET for EPR-1.

Calculation and Quantitation of Autoradiographic Signal

The signal generated from the mRNAs detected with the radiolabelled probe were quantitated using a laser scanner (Type JX 330, Sharp Ltd/Amersham Pharmacia Biotech Ltd, St Albans, UK) linked to Aida v2.00 image analysis software (Raytest Isotopenmessgerate GmbH, Germany). Absorbance values of the signal representing the bands for the mRNAs were normalised relative to loading of total RNA in the same sample. This was determined by either scanning the ethidium bromide stained 18S ribosomal RNA band in the gel photograph, or by performing Northern analysis for β-actin, a gene constitutively expressed by fibroblasts).

2.2.5 MICROSCOPY

2.2.5.1 Laser-Scanning Confocal Microscopy

The localisation of PAR-1 in human foetal fibroblasts was assessed by immunocytochemical staining and laser-scanning confocal microscopy.

Cell Culture Conditions

The cells were plated in DMEM-10% NCS at a density of 15000 cells/ 0.5 ml/ chamber in 8-chamber plastic tissue culture slides (Corning Costar Ltd, High Wycombe, UK). The cells were grown to 70% confluence in the slide chambers (72 hr), washed three times with PBS-0.1% bovine serum albumin (BSA) and fixed in cold 4% paraformaldehyde for 5 min. The fixed cells were kept in the dark at 4°C until staining.

Cell Surface Receptor Labelling: Primary Antibody

Before the addition of primary antibody, non-specific binding was avoided by blocking each chamber with newborn calf serum in PBS (PBS-5% NCS) for 1 hr at 4°C. Subsequently, the cells were washed three times with cold PBS-0.1% BSA. PAR-1 detection was performed by incubating fixed fibroblasts with a monoclonal anti-PAR-1 antibody (mAb WEDE15), 1:100 in PBS-0.1% BSA for 45 min at 4°C (primary antibody). This was followed by two washes with 1 ml of ice-cold PBS-0.1% BSA for each chamber. In negative controls, the specific primary antibody was substituted for non-specific mouse Immunoglobulin G_1 kappa (Sigma Chemicals Ltd, Poole, UK). No signal was observed when the primary antibody was omitted, or replaced with mouse Immunoglobulin G_1.

Fluorescent Labelling: Secondary Antibody

Primary mouse anti-PAR-1 antibodies were detected using a fluorescin-5-isothiocyanate (FITC)-conjugated goat anti-mouse antiserum, 1:200 in PBS-0.1% BSA (Sigma Chemicals Ltd, Poole, UK). Cells were incubated with the antibody for 45 min at 4°C. The labelled cells in each chamber were washed three times with cold PBS-0.1% BSA, immersed in Sigmacote mounting fluid (Sigma Chemicals Ltd, Poole, UK) and covered with a thin glass coverslip. The slides were stored in the dark at 4°C.

Visualisation

Slides were scanned using a confocal laser-scanning microscope linked to a TCS-NT microscopy system (Leica Lasertechnik GmbH, Heidelberg, Germany), as reported previously (Lakkakorpi *et al.,* 1993). Briefly, the FITC fluorochrome was excited by laser at 488 nm wavelength at which fluorescin emits a green fluorescence signal. Fluorescent images were collected in 1 μM steps through the fibroblasts, from the top to the bottom of the cells. The sectional scans

were accumulated to produce a comprehensive composite picture using an image analysis system (Bio-Rad MRC 600, Bio-Rad, Hemel Hempstead, UK).

2.2.5.2 Intracellular Calcium Spectrofluorometry

Cell Culture Conditions

The effect of proteases on intracellular calcium concentration ($[Ca^{2+}]_i$) was determined in human foetal lung fibroblasts (HFL-1), wild type and PAR-1-deficient mouse lung fibroblasts. Cells were plated in 4 ml of DMEM + 10 % NCS at a density of 5000 cells / ml in 50 mm diameter hydrophilic Petriperm tissue culture plates (Heraeus, Germany) and grown to 50% confluence.

Before measuring $[Ca^{2+}]_i$ changes in response to protease stimulation, the cells were pretreated with or without the relevant proteases for 90 min (including fluorescent dye labelling for the last 30 min) at 37°C and 5% CO_2 in 4 ml of hepes buffered saline (HBS). HBS is a minimal cell culture medium comprising 120 mM NaCl, 25 mM glucose, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 20 mM HEPES, pH 7.2. Incubation with selected proteases for 90 min was used to desensitise fibroblasts according to the method of Ellis and colleagues (1999), explained in section 'PAR-1 desensitisation' (section 5.1; table 2).

	Medium	Time
1) Cell culture	DMEM-5% NCS	24 hr
2) Rinse cells once	HBS	-
3) Preincubation ± 'Desensitisation'	HBS ± Pretreatment	1 hr
4) 'Spike' with Fura-2 AM and Ca^{2+} labelling	HBS ± Pretreatment + Fura-2 AM	30 min
5) Rinse cells twice with HBS and cover with HBS	HBS	-
6) Kinetics of $[Ca^{2+}]_i$	HBS + Treatment	4 min

Table 2 Flowchart of the calcium spectrofluorimetry protocol

This table shows the steps of cell preparation, loading with fluorescent dye and the sequence of pretreatment of the cells to obtain protease-activated receptor desensitisation before stimulation and monitoring of intracellular calcium concentration changes. HBS is hepes buffered saline.

Principle of Assay and Ca^{2+} Fluorescent Detection

To study $[Ca^{2+}]_i$ changes, the cells were loaded with the calcium fluorescent indicator Fura-2 AM. Fluorescent Fura-2 AM is a *ratio dye* used to localise and evaluate Ca^{2+} concentrations by measuring fluorescence after excitation at two wavelengths, 340 nm and 380 nm (examples in figure

2.4, page 75). The dye fluoresces maximally at 340 nm when chelating Ca^{2+}, or at 380 nm when free. The ratio of fluorescence intensity at the two wavelengths can be calculated to indicate the presence or absence of free cytosolic Ca^{2+}. The $[Ca^{2+}]_i$ evaluated against a calibrated control.

The cells were spiked with freshly prepared Fura-2 AM to a final concentration of 2 μM and incubated in HBS for 30 min at RT and 5% CO_2. Fura-2 AM (Molecular Probes Europe BV, Leiden, The Netherlands) is only partially soluble in H_2O, thus it was initially dissolved in 25 μl of dimethylsulphoxide dimethyl sulphoxide (Sigma Chemicals Ltd, Poole, UK) and further dissolved in HBS plus 5 mg/100ml Pluronic F-127 (Molecular Probes Europe BV, Leiden, The Netherlands). The fluorescent dye aliquots and the dye-loaded cells were kept in the dark at all times since excessive excitation by daylight would cause loss of fluorescence signal.

Visualisation

After loading with Fura-2 AM, the dishes were rinsed with HBS and placed on the stage of an Axiovert 100TV inverted microscope (Carl Zeiss Ltd, Welwyn Garden City, UK) fitted with Fucal 5.12c imaging system (TILL Photonics GmbH, Martinsreid, Germany) for acquisition of 340/380 nm ratio of pairs. A xenon arc lamp was used to give out light over a wide range of wavelengths and an automated unit moved two filters to transilluminate the plates at the desired wavelengths, 340 nm and 380 nm successively. A camera monitored the fluorescence output of cells at both wavelengths after each cycle for up to 4 min. One cycle lasted 2 sec. Photographs of resting and activated human fibroblasts are shown in picture 2. Panel A shows resting human fibroblasts labelled with Fura 2-AM after excitation at 340 nm and 380 nm. In resting cells, the fluorescence intensity is greater at 380 nm. Panel B shows the same cells 158 sec after stimulation with fXa (25 nM) and the maximal fluorescence intensity is greater at 340 nm after exposure to fXa (100 nM).

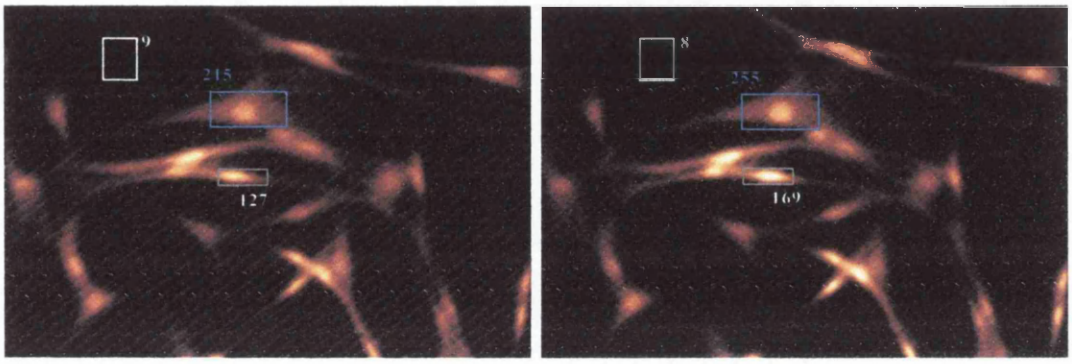
Delivery of Test Solutions

To reduce the volume of the test solutions required for each stimulation, a ring-shaped teflon spacer was inserted in the tissue culture dish and sealed down onto the dish filter with grease to create a perfusion chamber. Test solutions were delivered by continuous perfusion of the chamber with 2 ml to 3 ml of test solution (total). When limited supplies of agonist were available (about 100μl), test solutions were puffed onto the cells with a glass micropipette ('capillary' from Clark Electromechanical Instruments Ltd, UK) placed in close proximity of selected fibroblasts. A pressure of 200 Hpa was applied to the back of the micropipette with a Transjector 5246 automated injection system (Eppendorf GmbH, Hamburg, Germany).

Fibroblasts loaded with fura-2 AM

RESTING CELLS (Before perfusion)

λ=340nm λ=380nm

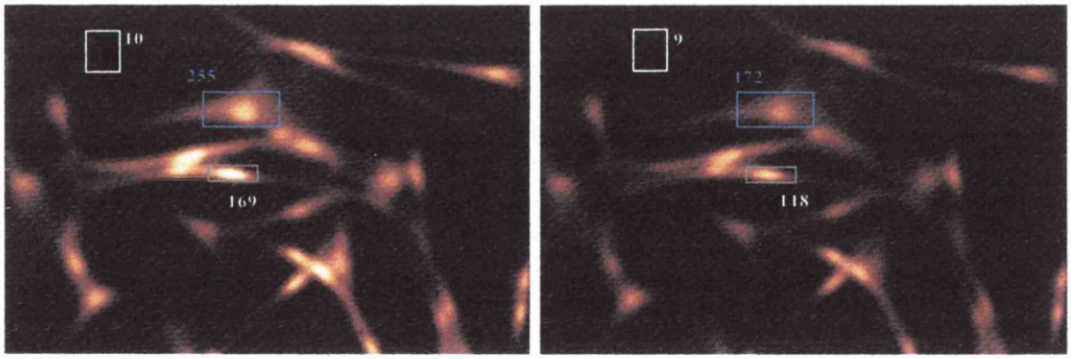


Background fluorescence has been substracted from each picture (white box):

Cell 1 (blue box) at rest: Ratio (215-9) / (255-8) = 0.83
Cell 2 (grey box) at rest: Ratio (127-9) / (169-8) = 0.74

ACTIVATED CELLS (158 seconds after perfusion with factor Xa)

λ=340nm λ=380nm



Perfusion of the cells with factor Xa increases the ratio $\lambda_{340} / \lambda_{380}$:

Cell 1 (blue box) activated: Ratio (255-10) / (172-9) = 1.50
Cell 2 (grey box) activated: Ratio (169-10) / (118-9) = 1.46

Figure 2.4 Example of cytosolic free Ca^{2+} measurement by fluorescence microscopy
Human foetal lung fibroblasts loaded with Fura-2 AM before and after exposure to fXa (100 nM). Background fluorescence is calculated in the white box and subtracted from the fluorescence measured in the grey and blue boxes that surround fibroblasts.

Selection of Suitable Cells

A field of 10 to 20 cells was chosen in the perfusion chamber and the body of each selected cell was defined by dragging a window around it in a preliminary picture. At this stage, cells with a basal $[Ca^{2+}]_i$ superior to 90 nM (ratio pair >1) were not retained. Such a high $[Ca^{2+}]_i$ was used as an indicator of cells already stimulated and mobilising Ca^{2+}, or with impaired $[Ca^{2+}]_i$ regulation. These were not considered suitable for the studies.

Calculation of Cytosolic Free Ca^{2+} Concentration

A kinetic of the $[Ca^{2+}]_i$ of each cell was generated and the data was translated by a digital converter and stored onto a personal computer. The image analysis software (Global Lab Inc, USA) was used to measure light emission form fibroblasts and analyse the image pairs. The system was calibrated at RT. At each wavelength, background signals were subtracted from cell signals. Finally, an estimation of the $[Ca^{2+}]_i$ was calculated by applying the formula below (Groden *et al.*, 1991):

$$[Ca^{2+}] = K_d^{Ca2+} \times S \times \left(\frac{R - R_{min}}{R_{max} - R} \right)$$

- $K_d^{Ca2+} = 236$ nM (Affinity constant of Fura-2 AM for Ca^{2+}, Groden *et al.*, 1991).

- The measured calibration parameters:

$$S = \frac{F_{340nm}\ min}{F_{340nm}\ max} = 5.17 \quad R_{min} = \frac{F_{340nm}\ min}{F_{380nm}\ min} = 0.48 \quad R_{max} = \frac{F_{340nm}\ max}{F_{380nm}\ max} = 8.3$$

- The measured test parameter: $R = \frac{F_{340nm}\ test}{F_{380nm}\ test}$ = measured for each sample at each cycle.

- F_{340nm} max and F_{380nm} max (saturated Fura-2): These values are fluorescence intensities at 340 nm and 380 nm produced by Fura-2 in the presence of 10 mM Ca^{2+}.

- F_{340nm} min and F_{380nm} min (free Fura-2): These values are fluorescence intensities at 340 nm and 380 nm produced by Fura-2 in the absence of Ca^{2+}.

2.2.6 FIBRINOGEN PEPTIDE PREPARATION

2.2.6.1 Fibrin Clot Formation *In Vitro*

FBN clots were produced *in vitro* by the interaction of human thrombin (6 nM) and (Sigma Chemicals Ltd, Poole, UK) with human FGN (3 μM; Sigma Chemicals Ltd, Poole, UK) dissolved in DMEM (pH 7.4) at RT for 45 min. After formation of a solid FBN gel, the newly formed clot was separated into a solid and a liquid phase by decantation. The liquid phase, termed *clot supernatant,* was collected in a polypropylene tube (Falcon, UK) and the insoluble FBN matrix was discarded. In order to remove microscopic clot fragments and possible contaminating micro-organisms, the clot supernatant was aspirated through a 19 gauge needle (Terumo Europe, Leuven, Belgium) and filtered through a 0.2 μm filter (Gelman Sciences, MI, USA).

2.2.6.2 Fibrinogen Chain Purification

Reduction and Alkylation of Fibrinogen

Human FGN (Sigma Chemicals Ltd, Poole, UK) was reduced and alkylated using methods described previously by Doolittle *et al.* (1977) and refined by Raut *et al.,* (1994). FGN was dissolved to a concentration of 7.3 μM in 20 ml of 6 M guanidinium chloride in the presence of 0.01 M dithiothreitol (both from BDH-Merk Ltd, Lutterworth, UK) and 0.2 M Tris buffer (FSA Ltd, UK), pH 8.5. This FGN solution was incubated at room temperature for 30 min and mixed with an equal volume of 6 M guanidinium chloride together with 0.03 M iodoacetic acid (Sigma Chemicals Ltd, Poole, UK). The carboxylation reaction was allowed for 30 min in the dark. The resulting solution was repeatedly dialysed against distilled water at 4°C for 24 hr. Dialysate was frozen at -40°C and lyophilised before use.

Separation of Reduced and Alkylated Fibrinogen Chains

Reduced and alkylated FGN chains were separated by cation exchange chromatography using a modification of a previous method (Doolittle *et al.,* 1977). A chromatography column XK-16 (Amersham Pharmacia Biotech Ltd, St Albans, UK; 1.4 cm in diameter, 16 in. long) was filled with CM sepharose CL-6B (Amersham Pharmacia Biotech Ltd, St Albans, UK). The sepharose was packed and equilibrated with 0.01 M sodium acetate (BDH-Merk Ltd, Lutterworth, UK), 8 M urea (Sigma Chemicals Ltd, Poole, UK), pH 5.2. About 75 mg of lyophilised, reduced and alkylated FGN

was dissolved in 10 ml of equilibration buffer and loaded onto the equilibrated column. A volume of 20 ml (just over 1 column volume) was passed through the column prior to the separation.

Separation of reduced and alkylated FGN chains was performed with a linear gradient of between 0.01 M to 0.18 M sodium acetate, 8 M urea, pH 5.2, to a final volume of 200 ml. The salt gradient was generated using a Pharmacia GM-1 gradient mixer (Amersham Pharmacia Biotech Ltd, St Albans, UK). A constant flow rate of 1 ml per min was maintained through the column using a peristaltic pump (Amersham Pharmacia Biotech Ltd, St Albans, UK). Eluent fractions were finally collected every five min in polypropylene tubes (Startedt Ltd, UK) loaded in a Pharmacia Heli rack fraction collector (Amersham Pharmacia Biotech Ltd, St Albans, UK). On completion of the gradient, it was necessary to pass through the column a further 20 ml of 0.22 M sodium acetate in 8 M urea, pH 5.2, to facilitate the elution of the Aα FGN chain.

Before being directed to the fraction collector, the eluant was passed through a UV flow cell spectrophotometer (Amersham Pharmacia Biotech Ltd, St Albans, UK). The protein concentration of the eluant was monitored in real time through the recording of its' absorbance at a wavelength of 280 nm. Elution profiles were plotted as histograms using a Pharmacia pen recorder (full scale deflection 0.2) connected to the UV monitor.

Concentration of Separated Fibrinogen Chain Batches

The three types of individual FGN chains (Aα, Bβ, and γ) were identified according to the order of elution (Doolittle *et al.*, 1977). The fractions containing each type of FGN chain were pooled and concentrated in Centriprep C-10 concentrators (Amicon Ltd, Stonehouse, UK) using a filter with a M_r cut-off of 10 kDa. The concentrator was loaded with FGN chain solution in the sample container and spun at 4,000 *g* for 40 min. Material with a M_r greater than the cut-off weight (FGN chains) remained in the sample container whereas smaller particles and the solvent would be forced through the filter into a filtrate container. The latter was emptied after each spin cycle to allow more FGN chain solution to be added to the sample container. This process was repeated until the entire volume of FGN chain solution was concentrated into a volume of 1 ml.

Bio-Rad Protein Concentration Assay

The concentration of each batch of FGN chain was determined with a Bio-Rad protein assay. This dye-binding assay (Bio-Rad, Hemel Hempstead, UK) is based on the differential colour change of the coomassie brilliant blue G-250 dye (CB G-250) when bound to protein and sensitive enough to measure accurately protein concentrations between 20 and 1400 μg/ml.

For each experiment, a standard curve was produced using several dilutions (0.2 to 1.4 mg/ml) of bovine serum albumin (BSA). Each solution was assayed by mixing thoroughly a 100 µl sample into 5 ml of dye reagent and allowed to stand for 30 min before absorbance was measured at 595 nm using a Guildford 2600 spectrophotometer (Guildford Instrument Laboratories Inc, USA). The absorbance produced by a protein blank containing 100 µl of buffer was used to zero the spectrophotometer. Finally, the absorbances of the standard dilution samples at 595 nm were plotted against protein concentration (µg of protein per 100 µl) and the slope of the curve was determined. Protein concentrations were calculated with the formula:

$$[\text{Protein}] \quad (\text{in } \mu g \text{ of protein per } 100\ \mu l) = \frac{\text{Absorbance (595 nm)}}{\text{Slope of standard curve}}$$

2.2.7 PHARMACOLOGICAL PROTEASE INHIBITION

2.2.7.1 Protease Inhibition *In Vitro*

FXa was incubated with rTAP or ASN for 2 hr at 37°C, with regular shaking, prior to addition to the cells in culture (inhibitor:enzyme ratios in legends). Catalytically inactive fXa was prepared by Dr C. Goodwin (National Heart and Lung Institute, London, UK) by incubating fXa (prepared in 0.5 mM $MgCl_2$, 10 mM Hepes, 0.15 M NaCl, 4 mM KCl, 11 mM glucose) with DEGR-ck with a ratio inhibitor:enzyme of 20:1. No significant catalytic activity remained after 2 min of incubation with the inhibitor, as measured by chromogenic assay with a specific synthetic substrate for fXa (S-2765 from Chromogenix Ltd, Epsom, UK). Excess DEGR-ck was removed by extensive dialysis. Inhibited fXa was termed DEGR-fXa.

2.2.7.2 Optimisation of Inhibitor and Antibody Concentrations

Prior to inhibition experiments, all protease inhibitors and antibodies were evaluated individually over a range of dilutions in the relevant assay. The maximal workable concentrations were determined to obtain optimal neutralisation at doses that do not interfere with basal cell functions. It has generally been found that incubation of fibroblasts with antibodies (mAb 5224, pAb JC15, mAb B6 and pAb anti-PDGF) for 48 hr affects mildly the basal absorbance levels of

cell layers in the methylene blue assay, possibly by enhancing fibroblast replication in a non-specific fashion. Thus it was felt that experiments with blocking antibodies should be performed in the DNA synthesis assay which allows the use of high concentrations of antibody without affecting basal ^{3}H-thymidine incorporation. Second, the neutralisation of the activity of fXa with antibodies was more effective in the DNA synthesis assay. The shorter incubation time in this assay may favour the kinetics of the inhibition with compounds such as antibodies, which do not bind convalently to their target, but compete for binding sites with natural ligands.

2.2.8 DATA AND STATISTICAL ANALYSIS

Data of this thesis are reported as mean ± standard error of the mean (s.e.m.). The number of repeat experiments and the number of replicate for each experimental value is also given.

Data are expressed either in the relevant unit for each assay or in percent increase above control. In these cases, the percent change relative to control cells treated without mediator, was calculated according to the equation below. When the data are expressed in percent increase above medium control, the control value has been subtracted from each experimental data. Thus the data represented is relative to the mean control value is arbitrarily considered as a basal level of 0% increase above control.

$$\% \text{ increase above control} = \left(\frac{\text{Value (treated)}}{\text{Value (control)}} - 1 \right) \times 100$$

Statistical evaluation was performed using an unpaired student *t* test for single group comparisons. For single parametric multiple comparisons, the analysis of variance test (ANOVA) was used (Statview version 5 software, SAS Institue Inc., USA). Fisher's PSLD statistical values for comparisons between groups were subsequently reported. In this thesis, the ANOVA test was employed for the statistical analysis of concentration:response curves. Assuming the null hypothesis to be correct, the mean values of measured parameters were considered to be significantly different when their probability to be similar fell below 5% (i.e.$p<0.05$).

CHAPTER 3

COAGULATION CASCADE PROTEASES STIMULATE FIBROBLAST PROLIFERATION

During normal tissue repair and in fibrotic diseases, the extrinsic coagulation pathway is the primary mechanism of activation of factors VII, IX, X and prothrombin (chapter 1).

In this chapter, it is hypothesised that the coagulation cascade serine proteases that are activated primarily through the extrinsic pathway are mitogenic to fibroblasts. This chapter will start with a review of the literature on these proteases. Second, the effects of factors VIIa, IXa, Xa and thrombin will be assessed on human foetal and adult fibroblast proliferation. Third, their mechanism of action will be investigated using specific catalytic site inhibitors and antibodies. The findings and their significance will be discussed at the end.

3.1 INTRODUCTION

This section will examine the biology of coagulation cascade proteases (reviewed in chapter 1). In particular, we will focus on the cellular effects of coagulation cascade proteases and their role during tissue repair and fibrosis.

3.1.1 THROMBIN

Thrombin catalyses FBN formation, during blood coagulation, but it also plays a major role in activating platelets, stimulating a variety of cell types at sites of tissue injury. It does so, at least in part, through the proteolytic activation of cell surface protease-activated receptors (PAR). Thrombin has been shown to activate three members of this rapidly growing family of receptors. They are PAR-1, the first PAR to be discovered (Vu *et al*, 1991; Coughlin *et al.*, 1993), PAR-3 (Ishihara *et al.*, 1997) and PAR-4 (Xu *et al.*, 1998). PAR-1, -3 and -4 mediate different functions of thrombin in a variety of cells and these receptors will be used to classify the effects of thrombin (PAR will be reviewed in section 4.1.2). The cellular effects of thrombin that are mediated via PAR-1 will be detailed first and the cellular effects mediated via PAR-3 and PAR-4 second.

3.1.1.1 PAR-1-Mediated Cellular Effects of Thrombin

Platelet Stimulation

In humans, thrombin activates PAR-1 on the surface of platelets and induces their aggregation (Liu *et al.*, 1997) and stimulates the secretion of several mediators, including thromboxane A2, platelet factor-4, PDGF (Hart *et al.*, 1990; Soma *et al.*, 1992) and TGF-β1 (Schini-Kerth *et al.*, 1997; Kahn *et al.*, 1999). Thus thrombin could indirectly control the activity of the cells surrounding the area of trauma by triggering the release of a great variety of mediators from platelets immediately after tissue injury. However, thrombin also has direct cellular effects on a variety of cell types, most of which are mediated via PAR-1 (Grand *et al.*, 1996 and Goldsack *et al.*, 1998 for review). Thrombin stimulates vascular permeability (Garcia *et al.*, 1995) and modulates the vascular tone via the stimulation of nitric oxide (NO), platelet activating factor and serotonin (Péry *et al*, 1998, for review).

Inflammation

Thrombin promotes inflammation by stimulating the production of interleukins in various cell types, including that of TNF-α, IL-1, IL-2, IL-6 and IL-8 (details in section 3.1.1.3 below). Thrombin is thought to stimulate a variety of inflammatory cell functions via the stimulation of these cytokines such as migration, (Drake *et al.*, 1992), activation (Naldini and Carney, 1996; Anrather *et al.*, 1997), secretion (Herbert *et al.*, 1990) and proliferation (Grand *et al.*, 1996 for review). For instance, B- and T-lymphocytes proliferate upon stimulation with thrombin via the stimulation of IL-2 production and IL-2 receptor expression (Naldini *et al.*, 1993). This up-regulation of IL-2 and IL-2r also stimulates natural killer activity in lymphocytes (Naldini and Carnet, 1996). In addition, thrombin enhances the pro-inflammatory effects of cytokines such as TNF-α (Anrather *et al.*, 1997).

Proliferation

Thrombin is mitogenic for a number of cell types including endothelial cells (Herbert *et al.*, 1994; Wang *et al.*, 1997) and smooth muscle cells (Bydlowski *et al.*, 1998), implying that it may contribute to vascular wall repair after injury. These effects are mediated in great part by PAR-1 activation (McNamara *et al.*, 1993) and additional non-proteolytic mechanisms that are still undefined (Bar-Shavit *et al.*, 1990; Herbert *et al.*, 1994). The transfection of smooth muscle cells with an antisense to PAR-1 inhibited the effects of the protease and down-regulated serum-

stimulated their proliferation (Chaikof *et al.,* 1995), suggesting two things: PAR-1 mediates the majority of thrombin-stimulated smooth muscle cell proliferation and that thrombin is one of the main mitogens present in serum.

Thrombin also enhances fibroblast migration (Dawes *et al.,* 1993), proliferation (Gray *et al.,* 1990; Ohba *et al.*, 1996) and procollagen synthesis (Chambers *et al.*, 1998). These effects can be mimicked by synthetic PAR-1-activating peptides based on the sequence of the tethered ligand of the receptor (Vu *et al.,* 1991; The details of PAR-1 biology are discussed in section 4.1). However, there is evidence that PAR-1-activating peptides do not always exactly reproduce the effects of thrombin on cells *in vitro.* Indeed, PAR-1 activating peptides are degraded by cellular proteolytic activity if they are not delivered Concurrently with aminopeptidase inhibitors (Zacharias *et al.*, 1993). The initial stimulus they provide before degradation is not sufficient to induce mitogenesis in a similar fashion to thrombin, suggesting that the long term effects of thrombin require the sustained activation of PAR-1. Thrombin induces mitogenesis if it stimulates the cells for at least 8 hr in glomerular epithelial cells (Zacharias *et al.*, 1993). Other studies showed a typical delay of 3 hr to 6 hr in the onset of thrombin-stimulated proliferation in smooth muscle cells compared to other growth factors such as PDGF or bFGF (Molloy *et al.*, 1996).

Extracellular Matrix Metabolism

Thrombin has also been reported to promote collagen deposition. It stimulates procollagen gene expression and biosynthesis in dermal (Michel and Harmand, 1990), dental pulp (Sundqvist *et al.*, 1995) and lung fibroblasts (Chambers *et al.*, 1998). This can be reproduced by PAR-1-activating peptides, suggesting that PAR-1 mediates these events (Chambers *et al.*, 1998). Furthermore, fibroblasts embedded in FBN matrices and exposed to thrombin increase their biosynthesis of collagens type I and III, while augmenting the ratio of collagen type III to type I (Michel, 1990). More collagen type III than type I is deposited during early tissue repair and thrombin may contribute to this event. Finally, collagen lattice contraction by fibroblasts*in vitro* is thought to mimic wound contraction and it is stimulated by thrombin (Pilcher *et al.*, 1994).

Thrombin also stimulates fibronectin deposition by mouse and chick embryonic fibroblasts embedded in a three dimensional collagen matrices (Kang *et al.,* 1991; Armstrong *et al.,* 1996). The secretion of fibronectin by fibroblasts correlates with increased mitogenesis, implying that these events are coupled in proliferating cells (Kang *et al.,* 1991). Furthermore, there is some evidence that PAR-1 activation by thrombin leads to increased proteoglycan synthesis in human endothelial cells (Peracchia *et al.,* 1994).

In addition, thrombin upregulates the expression of basement membrane ECM elements such as collagen type IV in human mesenglial and endothelial cells (Kaizuka *et al.*, 1999) as well as laminin deposition by endothelial cells (Papadimetriou *et al.*, 1997).

Taken together these observations suggest that thrombin and PAR-1 activation may contribute to connective tissue deposition after injury by promoting collagen, fibronectin, laminin and proteoglycan deposition at sites of tissue repair.

3.1.1.2 PAR-3- and PAR-4-Mediated Cellular Effects of Thrombin

There is less known about PAR-3 and PAR-4 than about PAR-1. These receptors have been identified on the surface of human and mouse platelets and their main function seems to be related to platelet biology.

In humans, PAR-1 is necessary for normal platelet aggregation upon thrombin stimulation and this can be blocked by using neutralising antibodies directed against the tethered ligand of PAR-1 (Liu *et al.*, 1997). These antibodies are effective to block the effect of moderate concentrations of thrombin. However, PAR-4 has been suggested to play a similar role for the activation of human platelets by thrombin, but activation of this receptor occurs only at higher concentrations of the protease (Kahn *et al.*, 1999). Recent evidence also suggest that the short peptide released upon PAR-1 cleavage by thrombin may also contribute to platelet activation (Furman *et al.*, 1998). This suggests that PAR-1 is necessary for human platelet activation by low concentrations of thrombin whereas PAR-4 provides a supplementary activation mechanism at greater thrombin concentrations (Kahn *et al.*, 1999).

In contrast with humans, mouse platelets lacking PAR-1 respond normally to thrombin (Connolly *et al.*, 1996). This observation incited a number of studies to identify the other thrombin receptors that mediate the effects of thrombin on rodent platelets. Recent studies have shown that both PAR-4 and PAR-3 are necessary to mediate normal mouse platelet aggregation (Kahn *et al.*, 1998). In addition, PAR-3-deficient mouse platelets respond to thrombin, but they do so with an extensive delay. Moreover, specific PAR-4-activating peptides mimic mouse platelet degranulation stimulated by thrombin (Xu *et al.*, 1998). This suggests that platelet aggregation is mediated predominantly by PAR-3 activation and platelet secretion via PAR-4 activation in mice.

Although the specific roles of these protease receptors have not been completely unravelled, these data suggest that physiological responses to thrombin may be mediated by different protease receptors in mice and humans.

3.1.1.3 Thrombin Stimulates Cytokine Production

The cellular effects of thrombin can be mediated directly through activation of PAR-1 or involve the release of secondary mediators. Proteolytic activation of PAR-1 is necessary for the proliferative effect of thrombin on fibroblasts (Trejo *et al.*, 1996). In fibroblastic synovial cells, the activation of PAR-1 was shown to lead to the release of PDGF-AB which then acts on the cells in an autocrine fashion to stimulate mitogenesis (Ohba *et al.*, 1996). Moreover, the up-regulation of PDGF by thrombin occurs at the mRNA level, as observed in endothelial cells (Garcia *et al.*, 1993) and vascular smooth muscle cells (Kanthou *et al.*, 1992). Consistent with the idea of PDGF acting as a secondary autocrine mediator, there is increased occupancy of the PDGF-α receptors in thrombin-stimulated smooth muscle cells (Bydlowski *et al.*, 1998) and a thrombin response element has recently been characterised in the PDGF-B chain gene promoter region in endothelial cells (Scarpati and DiCorleto, 1996). Thrombin also up-regulates both PDGF receptor α– and β–chain expression and mRNA levels (Ohba *et al.*, 1996). The additional stimulation of the cytokine and its' receptor may potentiate the effect of the protease and ensure that autocrine stimulation takes place.

In addition to the release of PDGF, thrombin stimulates other growth factors such as basic fibroblast growth factor (bFGF) in vascular smooth muscle cells (Samaniego *et al*, 1997) and megakaryocytes (Jones *et al.*, 1992), or endothelin-1 in endothelial cells (Kohno *et al.*, 1990). It has been suggested that bFGF production and autocrine stimulation contributes to vascular smooth muscle cell proliferation in response to thrombin (Weiss and Maduri, 1993). Thus, the mitogenic effects of thrombin are mediated by the release of several growth factors in different cell types via proteolytic and non-proteolytic mechanisms.

Thrombin has pro-inflammatory properties via the stimulation of interleukin-1 (IL-1) by macrophages (Jones and Greczy, 1990) and monocytes (Hoffman and Cooper, 1995b). When combined with endotoxin, thrombin also stimulates the production of the potent pro-inflammatory cytokine tumour necrosis factor-alpha (TNF-α) in monocytes (Hooffman and Cooper, 1995b). The effect of thrombin on IL-1 production involves PAR-1 activation, whereas the release of TNF-α occurs independently. IL-1 and TNF-α are early mediators of inflammation that can themselves stimulate the production of other interleukins, including IL-6 and IL-8 (Loppnow *et al.*, 1998; Carre and Leophonte, 1993, for review). Thrombin provokes IL-6 production by fibroblasts (Sower *et al.*, 1995), endothelial cells, smooth muscle cells, osteoblasts and other cells (Grand *et al.*, 1996, for review) via PAR-1 activation (Hou *et al.*, 1998).

Thrombin can also stimulate IL-8 production in lung type II epithelial cells in an IL-1- and TNF-α-independent manner (Kaplanski *et al.*, 1997). Furthermore, thrombin has been suggested to proteolytically modify IL-8 released by endothelial cells and leukocytes and create a number of IL-8 cleavage products with pro-inflammatory activity (Herbert *et al.*, 1990). Thrombin's effects on interleukin production are mainly independent from cell proliferation (Loppnow *et al.*, 1998).

Secondary growth factors and cytokines stimulated by thrombin can also stimulate the expression of PAR-1 via feedback mechanisms. PDGF-AB and TGF-β1 stimulate PAR-1 expression in vascular smooth muscle cells (Schini-Kerth *et al.*, 1997). Hus, the reciprocal stimulation of receptor expression creates regulatory loops through which thrombin and growth factors such as PDGF can potentiate and amplify each other's effects. Similarly to mesenchymal cell proliferation, the stimulation of procollagen synthesis by thrombin via PAR-1 activation in fibroblasts and smooth muscle cells seems to be mediated by an as yet unidentified secondary mediator (Chambers *et al.*, 1998; Dabbagh *et al.*, 1998).

Summary

Thrombin stimulates various pro-inflammatory cytokines and may promote the invasion of injured tissues by inflammatory cells, including monocytes, macrophages and neutrophils and lymphocytes. Furthermore, thrombin modulates fibroblast, smooth muscle and endothelial cell proliferation, migration and gene transcription *in vitro*.

3.1.2 Other Coagulation Cascade Proteases

In comparison with thrombin, there has relatively much less interest in the cellulareffects of other coagulation factors. These will be reviewed in the following sections.

3.1.2.1 Factor Xa

Inflammation

FXa stimulates the production of pro-inflammatory cytokines in various cells, including IL-1 by fibroblasts (Jones and Greczy, 1990), IL-2 by lymphocytes (Altieri *et al.*, 1994) as well as IL-6 and IL-8 by endothelial cells (Senden *et al.*, 1998). These effects appear to be mediated by the initial stimulation of NO production by endothelial cell and smooth muscle cells*in vitro* and *in vivo*. This affects local inflammatory reactions as well as the vascular tone since NO promotes

vasodilatation (Papapetropoulos *et al.*, 1998). FXa also induces oedema formation when injected subcutaneously in a rat paw inflammation model, via the local recruitment of mast cells (Cirino*et al.*, 1997). FXa has also been shown to stimulate smooth muscle cell migration (Sato *et al.* , 1997). Thus, fXa promotes inflammation and chemotaxis by stimulating NO and interleukin in vascular and stromal cells.

Proliferation

FXa stimulates smooth muscle cell DNA synthesis in via the autocrine PDGF stimulation (Gasic *et al*, 1992; Kho *et al.,* 1996). Furthermore, binding to EPR-1 on the surface of smooth muscle cells is necessary for PDGF production and proliferation in response to fXa (Herbert *et al.*, 1998). Similar observations were also obtained in endothelial cells (Gajdusek *et al.,* 1986). In addition, the catalytic site of fXa is essential for its' mitogenic effect since specific inhibitors such as tick anticoagulant peptide (TAP) abolish this effect on smooth muscle cells and endothelial cells (Kho *et al.,* 1996).

One study began to examine the effect of fXa on fibroblast proliferation. Human fXa has not been found to stimulate DNA synthesis in fibroblasts isolated from calf dermis or transformed mouse 3T3 fibroblasts, (Gasic *et al.,* 1992). In addition, fXa is mitogenic to lymphocytes and this effect is thought to be mediated via the release of IL-2 (Altieri and Stamnes, 1994). Subsequent studies suggested that fXa may play a role in the replication of Reed-Sternberg cells in Hodgkin's disease via this mechanism (Adida *et al.*, 1996).

Summary

There is some evidence that fXa regulates cellular activities. Most of these effects are mediated through binding to effector cell protease receptor-1 (EPR-1) on the surface of target cells (Stamnes and Altieri, 1994, Herbert *et al.,* 1998). In addition, most of the cellular effects of fXa are mediated by the release of potent cytokines and growth factors that subsequently stimulate cells in autocrine or paracrine mechanisms.

3.1.2.2 Factor XIIa

The intrinsic coagulation pathway protease factor XII in its zymogen and active form has been shown to be mitogenic to human hepatoma G2 cells and several other EGF-sensitive cells, including foetal hepatocytes, endothelial cells, lung alveolar type II cells and aortic smooth muscle

cells (Gordon *et al.*, 1996). Factor XIIa seems to induce its' effects via EGF repeats in its' sequence through an unknown mechanism. In summary, two groups of coagulation cascade proteases exhibit mitogenic properties in smooth muscle cells and endothelial cells. Thrombin and fXa stimulate their effects via their catalytic sites and factor XIIa via its' EGF repeats. However, the effect of factor XIIa on fibroblasts remains unknown.

3.1.3 Summary and Aims of this Chapter

3.1.3.1 Summary

The biology of coagulation cascade proteases has been reviewed with a particular focus on their effects on fibroblast function. This led to the following conclusions:

- Thrombin is a potent stimulator of fibroblast proliferation and collagen synthesis *in vitro*. Thus thrombin may contribute to connective tissue formation through the direct stimulation of fibroblast ECM deposition and proliferation.

- The vitamin K-dependent proteases factor VII, IX and X share strong homologies with prothrombin, but the effects of factors VIIa, IXa and Xa on fibroblasts are unknown.

- There is increasing evidence that fXa and thrombin stimulate similar cellular functions such as cytokine production and proliferation in vascular cells, but one previous study suggested that fXa, unlike thrombin, is not mitogenic for fibroblasts.

3.1.3.2 Specific Hypothesis of this Chapter

The hypothesis on which the studies in this chapter are based is that:

> The coagulation proteases factors VIIa, IXa and Xa are mitogenic to fibroblasts.

3.1.3.3 Aims of this Chapter

In order to address this hypothesis, this chapter has the following specific aims:

- To assess the mitogenic potential of coagulation cascade proteases factors VIIa, IXa and Xa on cultured fibroblasts using DNA synthesis and proliferation assays and characterise their effects in terms of potency and kinetics.

- To determine whether factors VIIa, IXa and Xa stimulate the release of mitogenic secondary mediators by fibroblasts using specific inhibitors and antibodies directed against selected growth factors.

3.2 RESULTS

To address he previous hypothesis, the effects of coagulation factors VIIa, IXa, Xa and thrombin were assessed and characterised in several assays measuring fibroblast DNA synthesis and proliferation.

3.2.1 COAGULATION CASCADE PROTEASES AND FIBROBLAST PROLIFERATION

The mitogenic effects of factors VIIa, IXa and Xa on human foetal lung fibroblast DNA synthesis (after 20 hr of incubation) and proliferation (after 48 hr of incubation) were measured in the absence of serum. For each protease, two figures are presented: One showing its' effect on fibroblast DNA synthesis and one on fibroblast proliferation. Human foetal lung fibroblasts will be used throughout this thesis, except when stated otherwise.

3.2.1.1 Effect of Factor Xa on Human Fetal Fibroblast Proliferation

FXa stimulated fibroblast DNA synthesis in a dose dependent fashion between 1 nM and 200 nM ($p<0.01$), stimulating a maximal increase of up to 245±40% above medium control at 200 nM (figure 3.1, panel A). FXa also stimulated fibroblast DNA synthesis in a dose dependent

fashion between 1 nM and 200 nM ($p<0.01$), stimulating a maximal increase of up to 175±9% above medium control at 100 nM (figure 3.1, panel B). After 48hr of incubation, high concentrations of fXa (>100 nM) seemed to cause some fibroblasts to lift off their substratum in the methylene blue assay in a manner reminiscent of trypsin.

3.2.1.2 Effect of Factor IXa on Human Fetal Fibroblast Proliferation

Factor IXa stimulated fibroblast DNA synthesis at 1000 nM ($p<0.01$), stimulating an increase of 76±7% above medium control (figure 3.2, panel A). Factor IXa also stimulated fibroblast proliferation between 125 nM and 1000 nM ($p<0.01$), stimulating a maximal increase of up to 26±4% above medium control at 1000 nM (figure 3.2, panel B).

3.2.1.3 Effect of Factor VIIa on Human Fetal Fibroblast Proliferation

Factor VIIa stimulated fibroblast DNA synthesis between 12.5 and 100 nM ($p<0.01$), stimulating a maximal increase of up to 242±47% above medium control at 100 nM (figure 3.3, panel A). The maximal stimulatory effect of the protease only compared to that of fXa at a similar concentration. Factor VIIa stimulated fibroblast proliferation between 1 and 100 nM ($p<0.01$), stimulating a maximal increase of up to 34±2% above medium control at 100 nM (figure 3.3, panel B).

3.2.1.4 Comparison of the mitogenic Effects of Factors VIIa, IXa and Xa with Thrombin and Platelet-derived Growth Factor

The proliferative effects of factors VIIa, IXa and Xa were compared with that of thrombin and PDGF in the proliferation assay (figure 3..4, panel A) and the DNA synthesis assay (figure 3..4, panel B). Identical experimental conditions were used in these assays. The proteases were used at 25 nM, a concentration of fXa and thrombin that falls below the physiological concentrations of their precursors in the circulation (140 nM and 1.4 μM, respectively; MacKie, 1996). To verify the mitogenic effect of the proteases independently from the effects on protein or DNA synthesis, direct cell counts were also performed (figure 3..4, panel C). Cell numbers were determined after incubation with the proteases in experimental conditions matching that used for the proliferation and DNA synthesis assays.

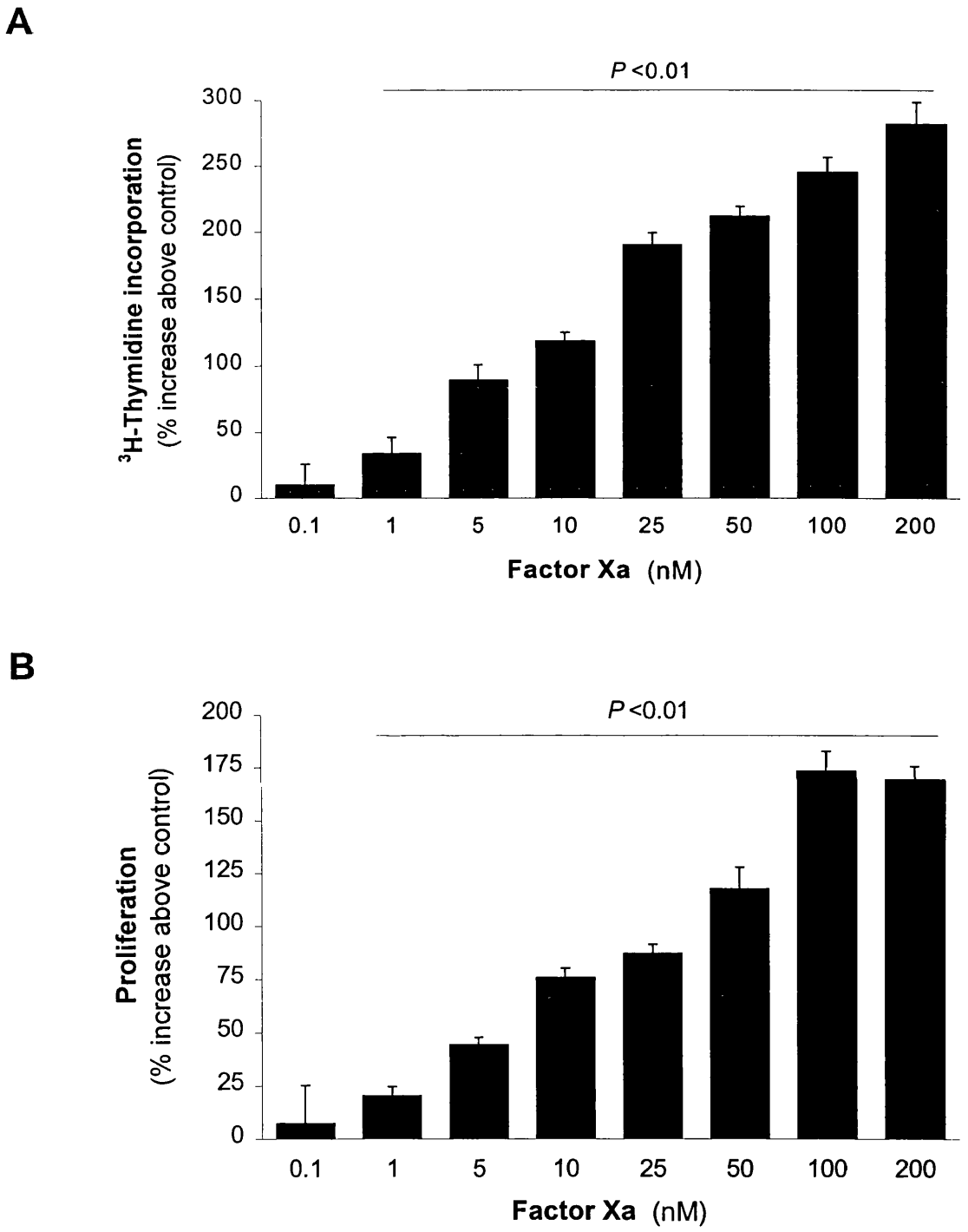


Figure 3.1 **Factor Xa stimulates human fetal lung fibroblast DNA synthesis and proliferation**

A ^{3}H-thymidine uptake assay; **B** Methylene blue colorimetric assay; representative experiments with 6 replicates for each value; expressed in % increase above control ± s.e.m..

For comparison, the positive control (10% serum) stimulated DNA synthesis by about 400% and proliferation by about 300 % in these assays.

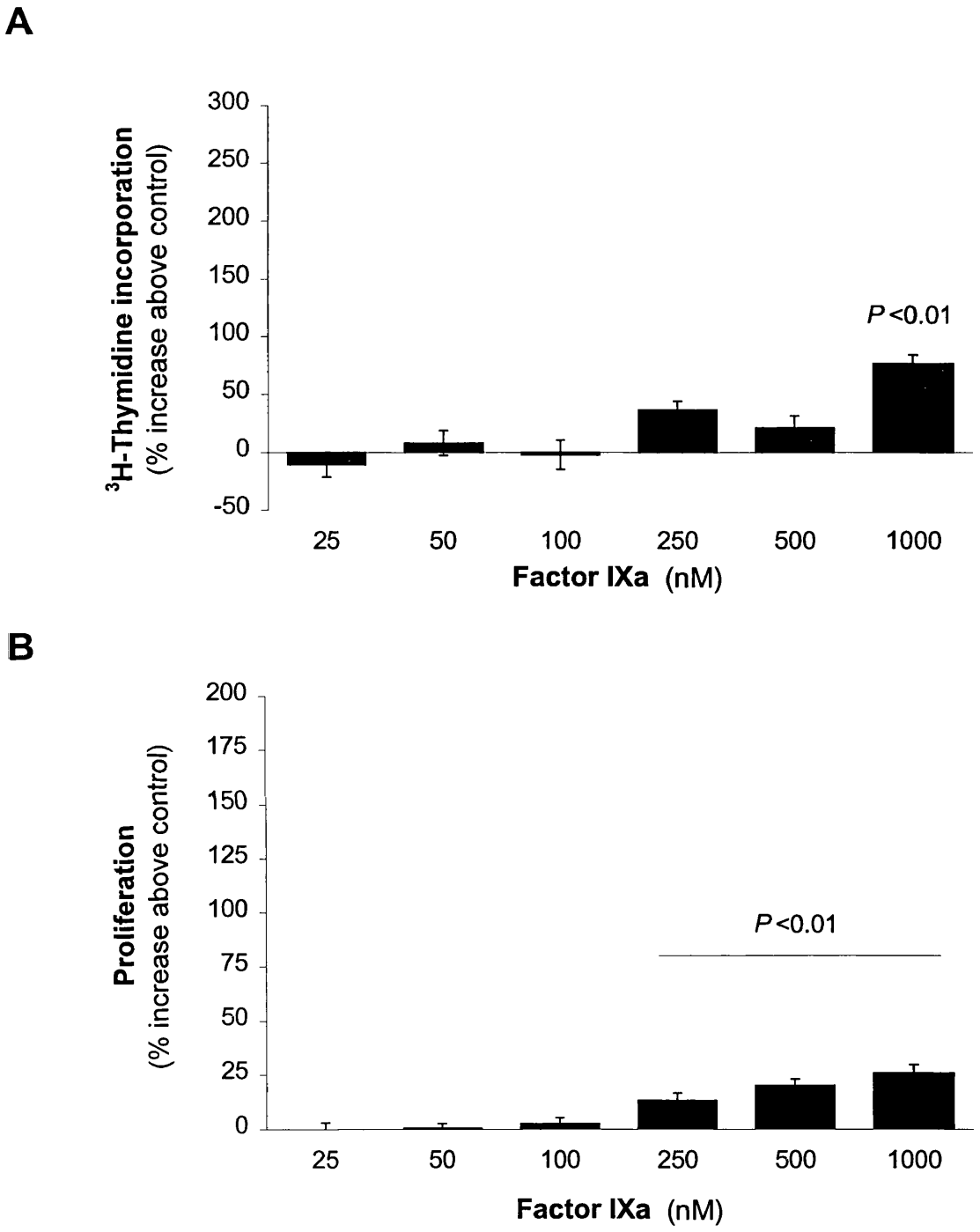


Figure 3.2 **Factor IXa stimulates human fetal lung fibroblast DNA synthesis and proliferation**

A ^{3}H-thymidine uptake assay; **B** Methylene blue colorimetric assay; representative experiments with 6 replicates for each value; expressed in % increase above control ± s.e.m.

The scales indicating increased proliferation or DNA synthesis have been conserved throughout these graphs to allow easy comparisons of the results.

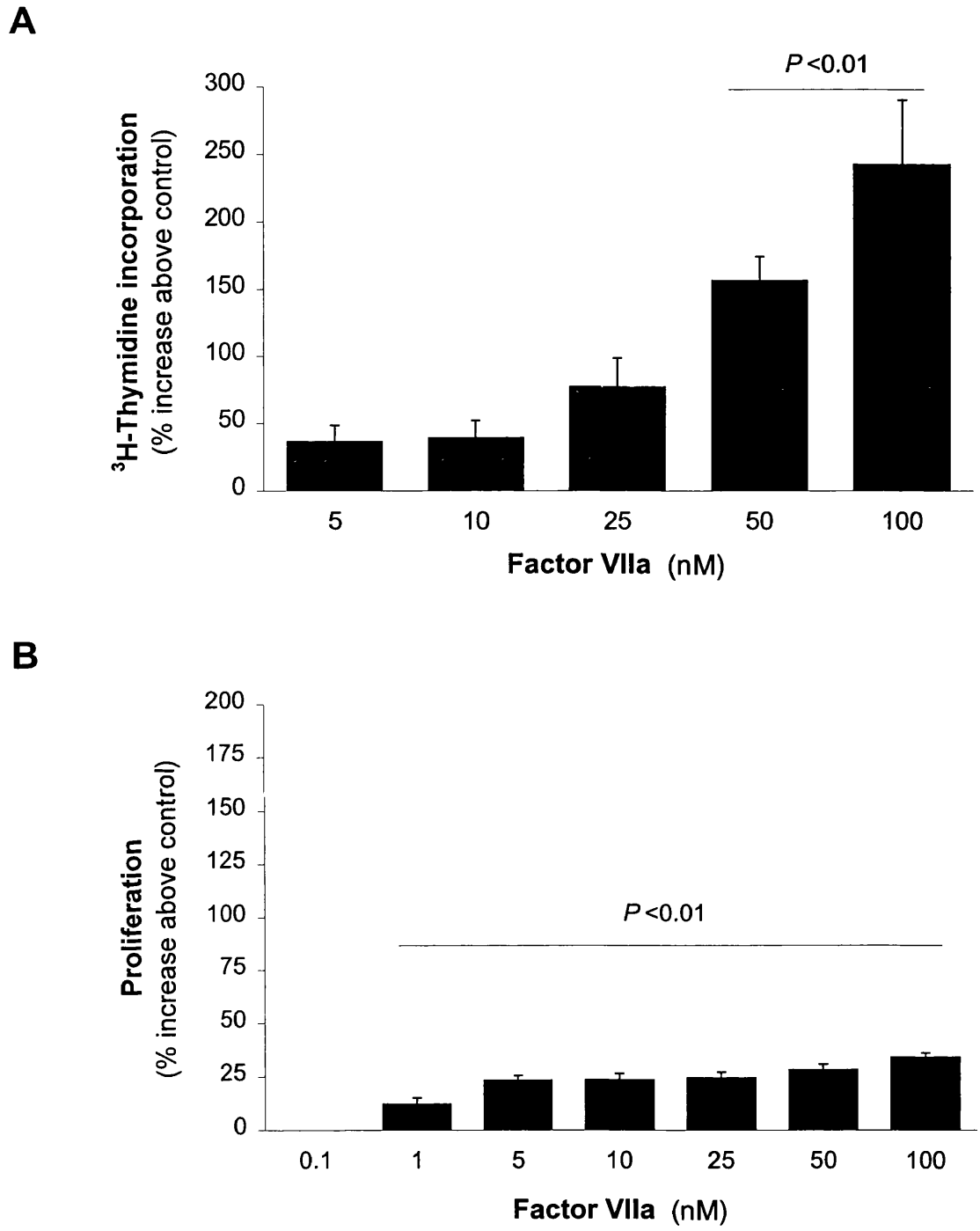


Figure 3.3 **Factor VIIa stimulates human fetal lung fibroblast DNA synthesis and proliferation**

A ^{3}H-thymidine uptake assay; **B** Methylene blue colorimetric assay; representative experiments with 6 replicates for each value; expressed in % increase above control ± s.e.m.,

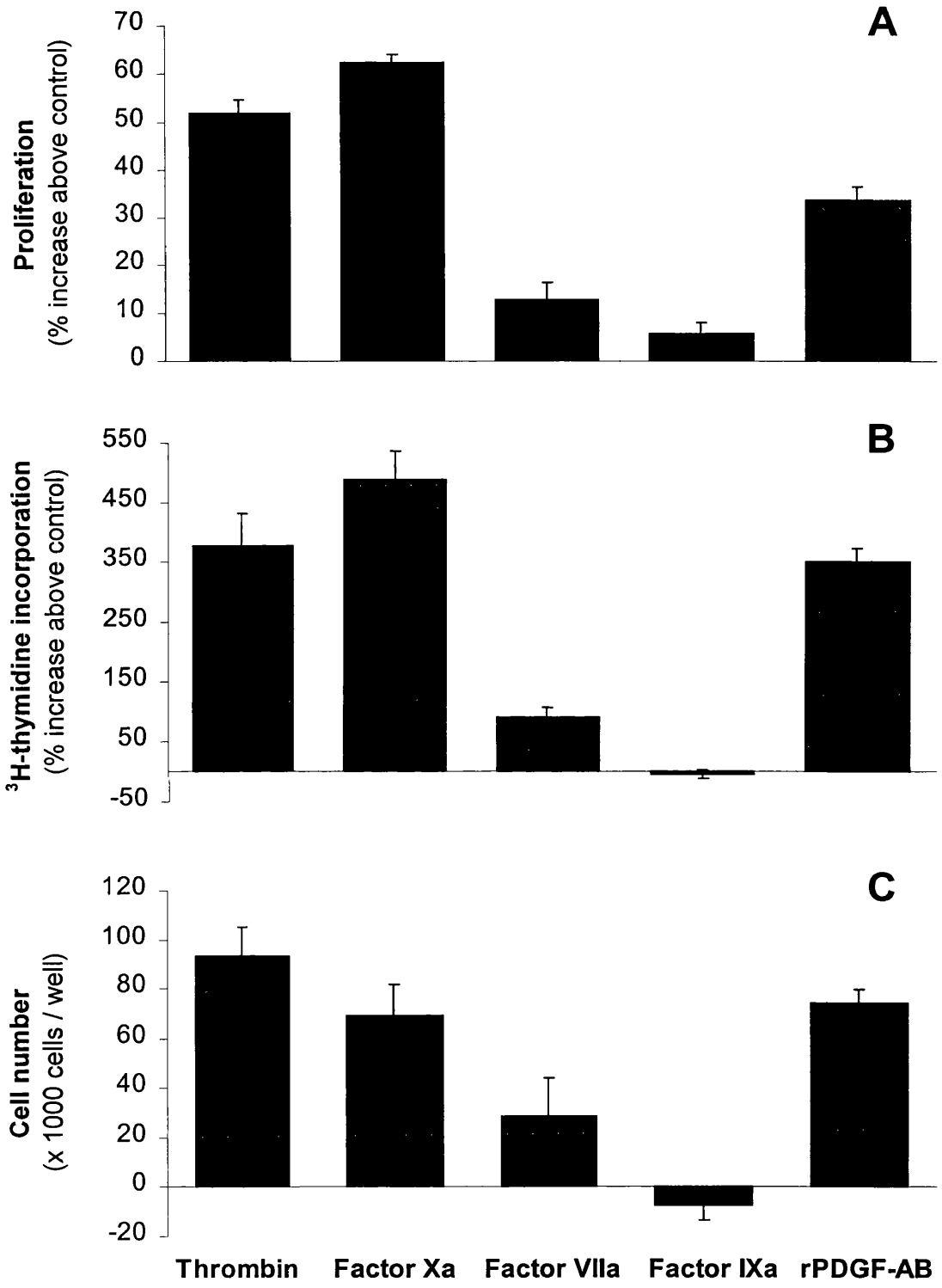


Figure 3.4 **Human fetal lung fibroblast proliferation in response to thrombin, factors Xa, VIIa, IXa and rPDGF-AB**

A Methylene blue colorimetric assay; **B** ^{3}H-thymidine uptake assay; **C** Direct cell counts. Representative experiments with 6 replicates for each value, data is mean ± s.e.m.. [Thrombin] = [factor Xa] = [factor VIIa] = [factor IXa] = 25 nM. [rPDGF-AB] = 0.4 nM.

3.2.1.5 Effect of Factor Xa on Human Adult Fibroblast Proliferation

The mitogenic effect of fXa was measured in the proliferation assay on primary cultures of fibroblasts freshly derived from explants of a range of normal adult human tissues. Table 3 shows that fXa was a potent mitogen for human primary fibroblasts isolated from normal adult skin, lung, heart and kidney, as well as foetal lung fibroblasts. However, foetal fibroblasts showed the greatest proliferative response to fXa and primary human adult fibroblasts displayed a proliferative response to fXa of about half of that of human foetal fibroblasts.

	Factor Xa (25 nM)
Foetal lung fibroblasts	92.3 ± 4.6 %
Primary adult **lung** fibroblasts	45.8 ± 3.0 %
Primary adult **skin** fibroblasts	35.7 ± 1.3 %
Primary adult **heart** fibroblasts	39.9 ± 1.2%
(SV40 transfd) adult **kidney** fibroblasts	55.7 ± 6.5%

Table 3 **Effect of factor Xa on the proliferation of normal human adult fibroblasts**
Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., representative experiments with 6 replicates for each value.
For comparison, the positive control (10% serum) stimulated fibroblast proliferation by about 300 %.

3.2.1.6 Commercial Factor Xa Preparations have Similar Mitogenic Effects

To select a preparation of fXa for this thesis, the mitogenic effects of two commercial preparations of fXa were assessed: One was obtained from Calbiochem Co. (La Jolla, CA, USA) and one from (American Diagnostica Inc., Greenwich, CT, USA).

Figure 3.5 shows that the two fXa preparations stimulated fibroblast proliferation with similar magnitudes at similar concentrations. There was no significant difference in potencies between preparations (p>0.05 between preparations at all concentrations tested).

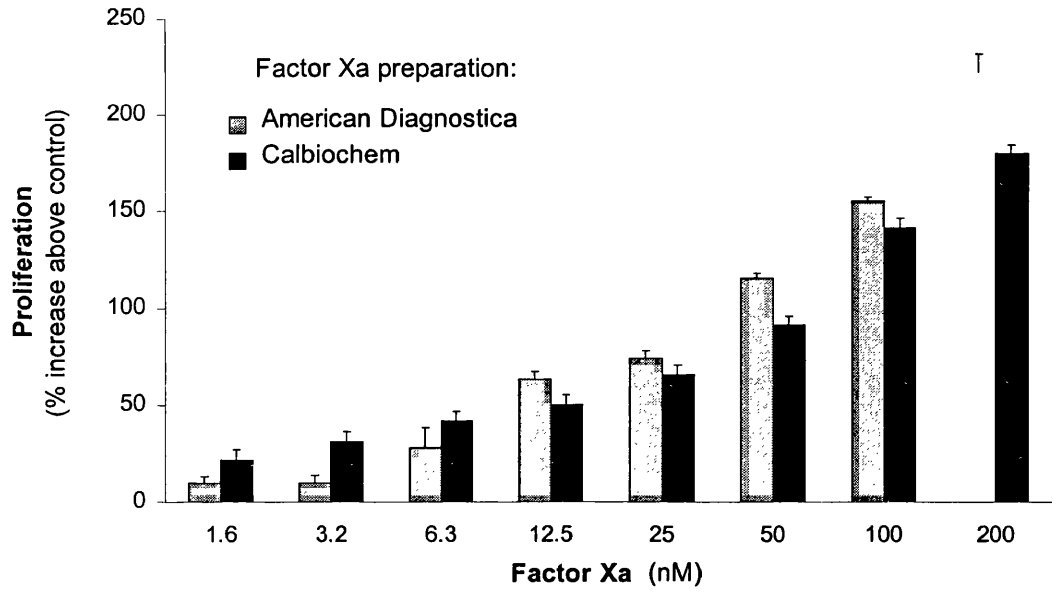


Figure 3.5 **Different commercial preparations of factor Xa stimulate human fetal lung fibroblast proliferation equally**

Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., representative experiment with 6 replicates for each value.

3.2.1.7 Kinetics of Fibroblast Proliferation Stimulated with Factor Xa

The kinetics of fibroblast proliferation in response to fXa and thrombin were determined. Figure 3.7 shows that fXa and thrombin (25 nM) stimulated fibroblast proliferation from 16 hr onwards. These effects were indistinguishable at any time points (p>0.1). Fibroblasts treated with culture medium only did not proliferate significantly over the 72 hr test period (section 5.3.3.2 for more evidence). Finally, serum (10% NCS) stimulated fibroblast proliferation significantly at all time points (table 4, next page).

There was no statistical difference between the levels of fibroblast proliferation stimulated by fXa or thrombin at 48, 64 and 72 hr (p>0.5). The levels of fibroblast proliferation reached a plateau after 64 hr of incubation and the optimal time point to observe the mitogenic potential of thrombin or fXa was between 48 hr and 64 hr.

The following section aims to begin to unravel the mode of action of fXa on fibroblasts. The regions of the protease that mediate its' mitogenic effects will be identified. The roles of the catalytic site and the conformation of the protease will be determined next.

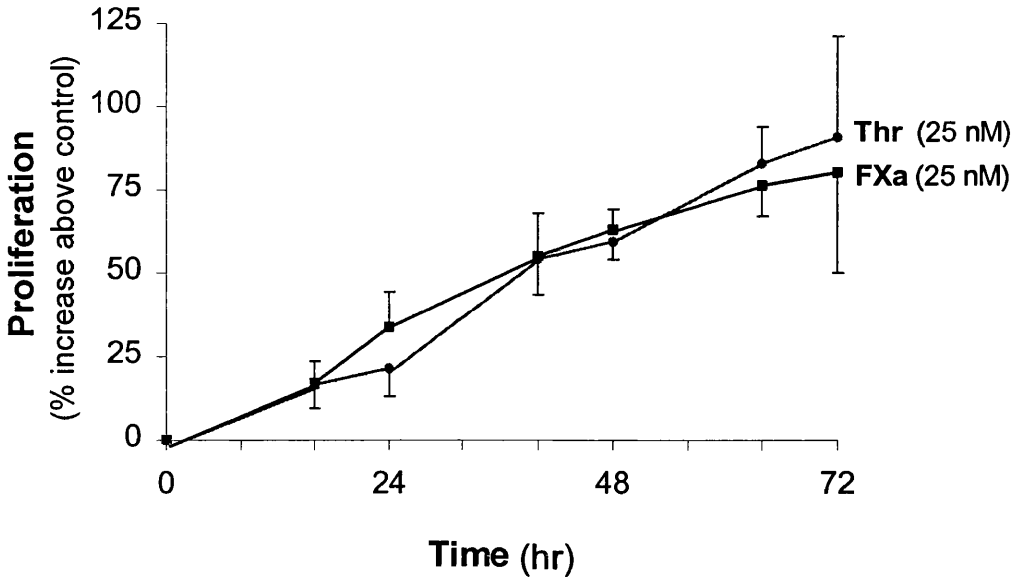


Figure 3.6 **Time course of factor Xa- and thrombin-induced fibroblast proliferation**
Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., *p* values are calculated between the effects of the two enzymes at each time point, representative experiment with 6 replicates for each value.

Time (hr)	16	24	40	48	64	72
Proliferation(% increase above control)	161	181	264	344	438	580
mean ± s.e.m.	± 8	± 11	± 11	± 2	± 18	± 14

Table 4 **Time course of the effect of serum (10% NCS) on the proliferation of human fetal lung fibroblasts**
Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., representative experiments with 6 replicates for each value.

3.2.2 Mechanism of Action of Factor Xa in the Stimulation of Fibroblast Proliferation

In this section, the role of the catalytic site of fXa will be determined using three groups of catalytic site inhibitors: a) a monoclonal antibody with neutralising properties, b) polypeptides mimicking the active region of potent natural inhibitors of fXa, c) a synthetic, small molecular weight compound. Second, the effect of the zymogen factor X on fibroblast proliferation will be assessed to determine the influence of its' conformation on its' mitogenic effects. Third, the

contribution of thrombin to the mitogenic effect of fXa will be determined using a specific thrombin inhibitor. Finally, the role of autocrine PDGF stimulation will be investigated using specific PDGF-neutralising antibodies.

3.2.2.1 Effect of a Specific Anti-Factor Xa Antibody

The effect of fXa on fibroblast DNA synthesis was determined in the presence or absence of a neutralising monoclonal antibody directed against the catalytic site of fXa. Figure 3.7 shows that mAb 5224 inhibited the mitogenic activity of fXa from 140±13% to 44±14% above medium control in a ^{3}H-Thymidine uptake assay.

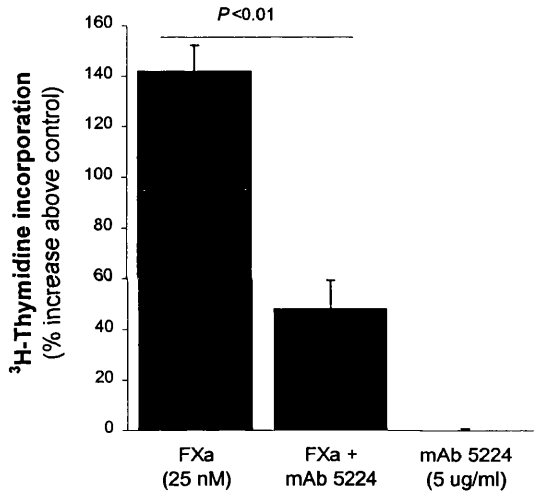


Figure 3.7 **Antibody 5224 inhibits factor Xa-induced fibroblast DNA synthesis**
^{3}H-thymidine uptake assay; expressed in % increase above control ± s.e.m., *p* values are calculated in comparison with control, representative experiment with 9 replicates for each value.

3.2.2.2 Effect of a Small Molecular Weight Inhibitor

DEGR-ck (dansyl-Glu-Gly-Arg chloromethyl ketone) is a small molecular weight compound that binds covalently to the catalytic site of fXa and inhibits its' activity irreversibly (section 2.1.2). A form of catalytically inhibited fXa, DEGR-fXa, was prepared and purified. Figure 3.8 shows that DEGR-fXa did not stimulate fibroblast proliferation.

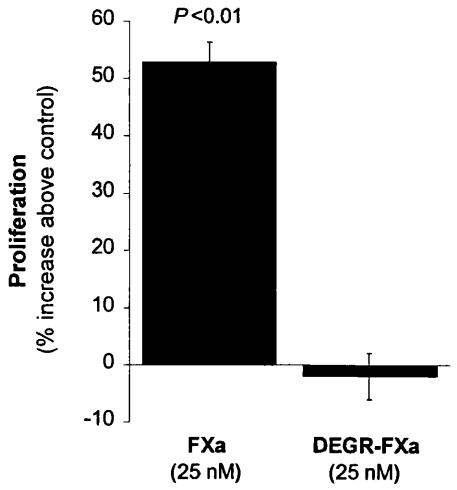


Figure 3.8 **Catalytically inactive factor Xa (DEGR-factor Xa) does not stimulate fibroblast proliferation**

Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., *p* value is calculated in comparison with control, representative experiment with 6 replicates for each value.

3.2.2.3 Effect of Polypeptides Mimicking Natural Inhibitors

Recombinant tick anticoagulant peptide (rTAP) and a synthetic peptide that mimics the core inhibitory region of leech antistasin (ASN related peptide) were used to abolish the mitogenic effects of fXa. These peptides inhibit fXa by binding to its' catalytic site with great affinity, but in a reversible fashion (section 2.1.2 for details).

Figure 3.9 shows that rTAP completely inhibited the mitogenic effect of fXa on fibroblasts. Complete inhibition was obtained with nanomolar concentrations of the inhibitor (molecular ratio inhibitor:enzyme = 5:1 or 10:1).

Figure 3.10 shows that ASN core peptide (ASN D-Arg^{32}-Pro^{38}) inhibited fXa mitogenic effect on fibroblasts. Concentrations of inhibitor in molar excess to the protease were necessary to obtain this effect (molecular ratio inhibitor:enzyme = 8:1, or 16:1).

3.2.2.4 The Mitogenic Effect of Factor Xa is not Mediated by Thrombin

To determine whether the mitogenic effect of fXa on fibroblasts is due to the local generation of thrombin, or contamination of the fXa preparation, the effect of fXa was determined in the presence of the specific thrombin inhibitor, hirudin. Figure 3.11 shows that hirudin had no effect on the proliferative effect of two different fXa preparations, but it inhibited over 85% of the

stimulation of thrombin. The fXa preparation from Calbiochem was selected for all subsequent experiments.

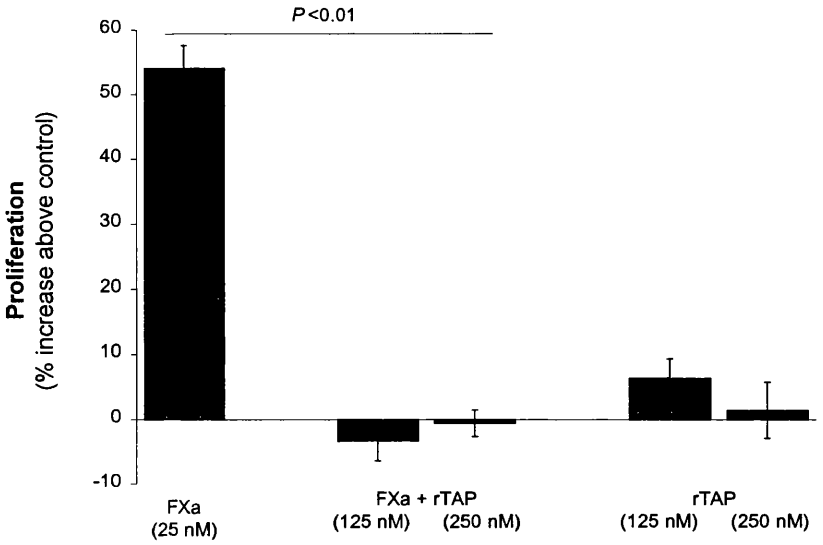


Figure 3.9 **rTAP inhibits factor Xa-induced fibroblast proliferation**

Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., *p* values are calculated between treatments, mean of 2 independent experiments with 6 replicates for each value.

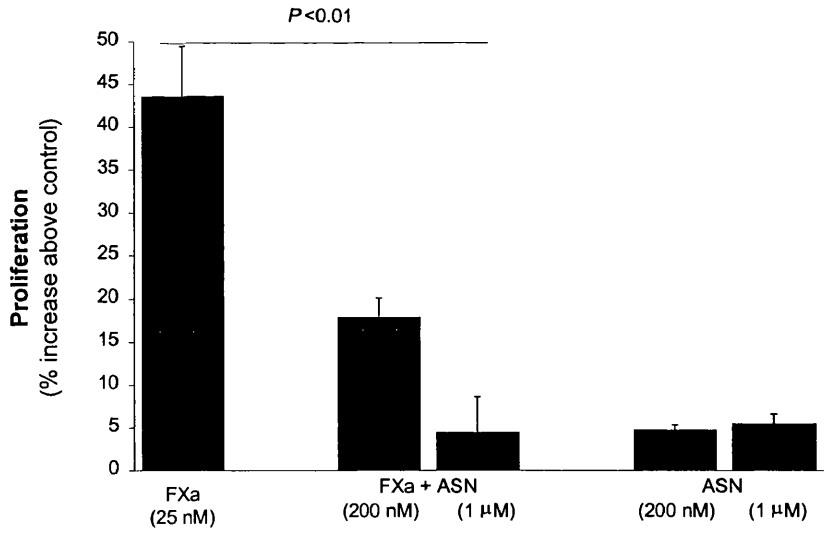


Figure 3.10 **ASN D-Arg^{32}-Pro^{38} inhibits factor Xa-induced fibroblast proliferation**

Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., *p* values are calculated between treatments, representative experiment with 6 replicates for each value.

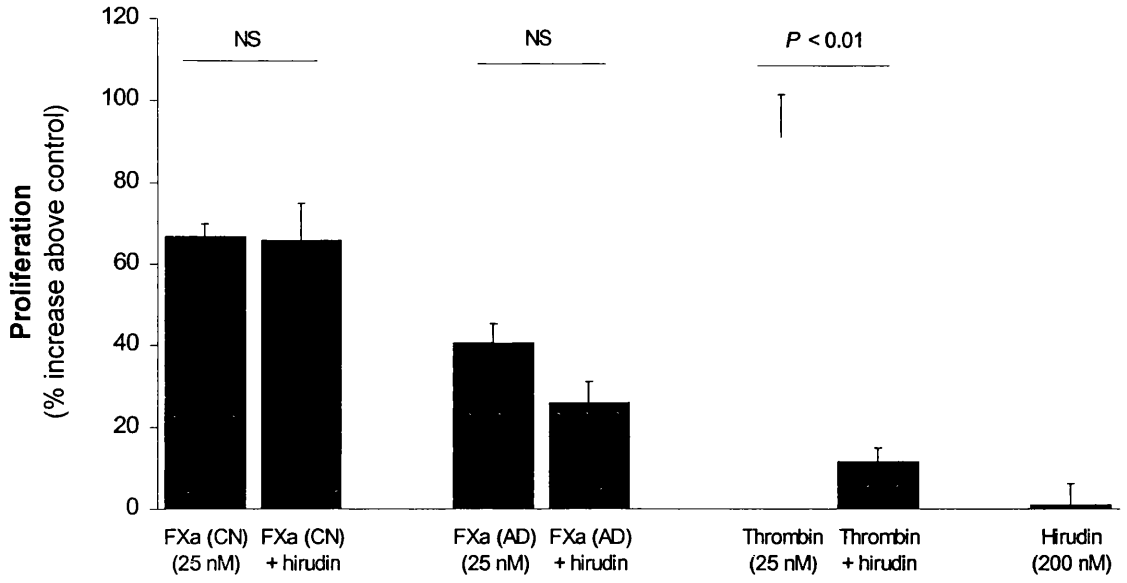


Figure 3.11 Hirudin did not inhibit factor Xa-stimulated proliferation human fetal lung fibroblasts.

Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., *p* values are calculated between stimulations with in the presence or absence of hirudin, representative experiment with 6 replicates for each value. CN: Calbiochem-Novabiochem (La Jolla, CA, USA); AD: American Diagnostica Inc. (Greenwich, CT, USA).

3.2.2.5 Effect of Factor X on Fibroblast Proliferation

Figure 3.12 shows the effects of the zymogen factor X on fibroblast DNA synthesis and proliferation were determined. Factor X stimulated fibroblast DNA synthesis between 50 nM and 100 nM, with a maximal increase of 131±7% above medium control at 50 nM (panel A). Factor X also stimulated fibroblast proliferation between 10 and 100 nM, with a maximal increase of 29±6% above medium control at 50 nM (panel B).

The effect of the active protease fXa was assayed at its' maximal stimulatory concentration (100 nM) in the same experiments. The maximal stimulation elicited by factor X amounted to only about 15% of that of fXa (figure 3.1 panel B, page 91, for comparison).

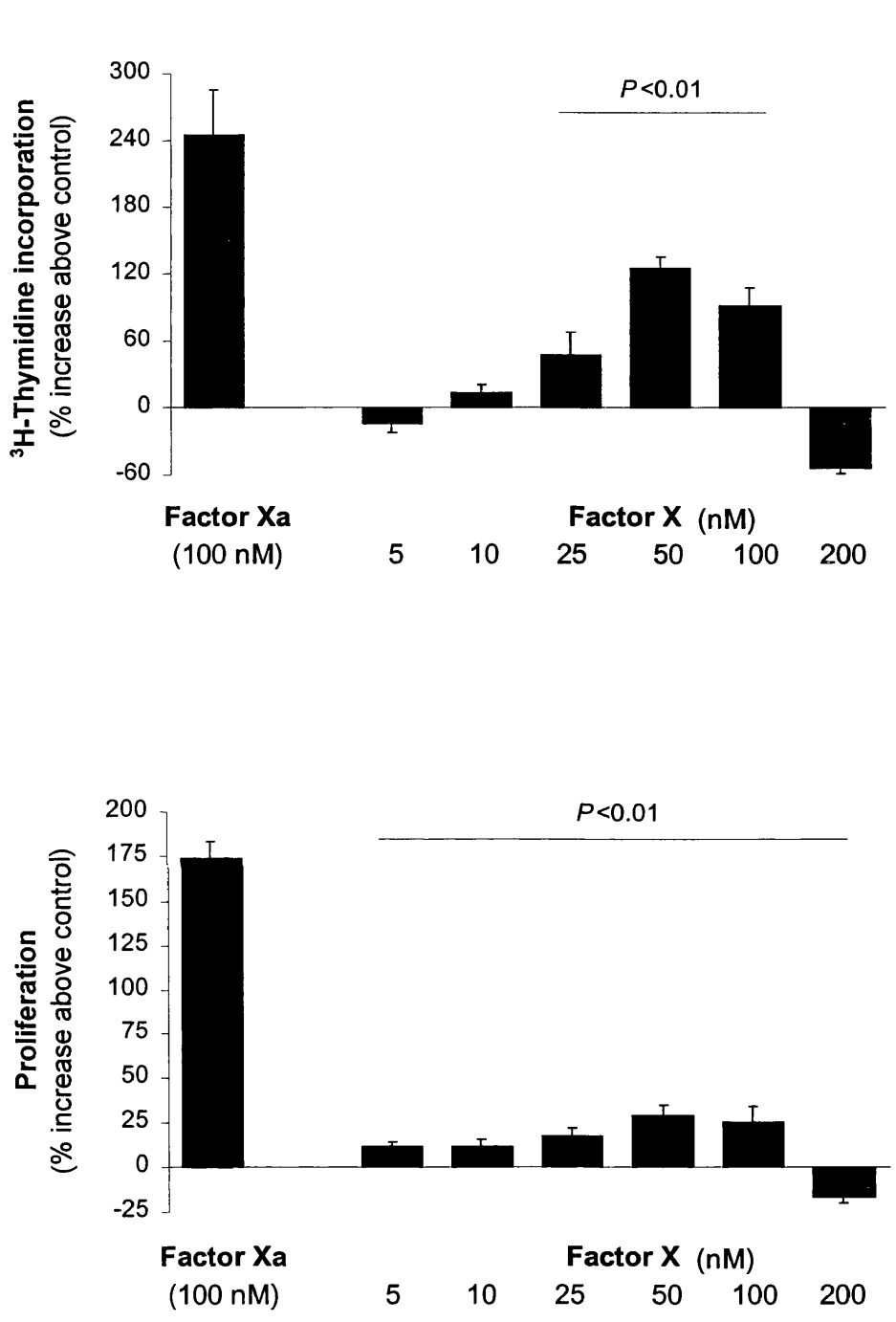


Figure 3.12 **Factor X stimulates human foetal lung fibroblast DNA synthesis and proliferation**

A ^{3}H-thymidine uptake assay; **B** Methylene blue colorimetric assay; representative experiment with 6 replicates for each value; expressed in % increase above control ± s.e.m., *p* value is calculated in comparison with control.

3.2.2.6 The Mitogenic Effects of Factor Xa and Thrombin are Additive

In order to determine whether the effect of fXa and thrombin are additive or synergistic, the proliferative effects of thrombin and fXa were assessed individually at two concentrations (25 nM and 50 nM) and in combination at a concentration of 25 nM.

Figure 3.13 shows that fXa and thrombin at 25 nM stimulated a similar increase in proliferation (46±7% and 50±9% above medium control respectively). There was no difference between their stimulations (p>0.67). At 50 nM, thrombin and fXa also induced similar increases in proliferation (87±7% and 87±8% above medium control) and there was no difference between these effects (p>0.99). The effect of serum (10% NCS) was measured as a positive control (358±87% above medium control) which confirmed that the cells had the potential to proliferate above the levels stimulated by fXa and thrombin at 50 nM. Furthermore, fXa and thrombin in combination (25 nM each) stimulated fibroblast proliferation to a level equal to that stimulated by thrombin or fXa individually at 50 nM (86±5% above medium control). There was no difference between any of these three stimulations (p>0.89 and p>0.91 respectively). The effects of the proteases are exactly additive.

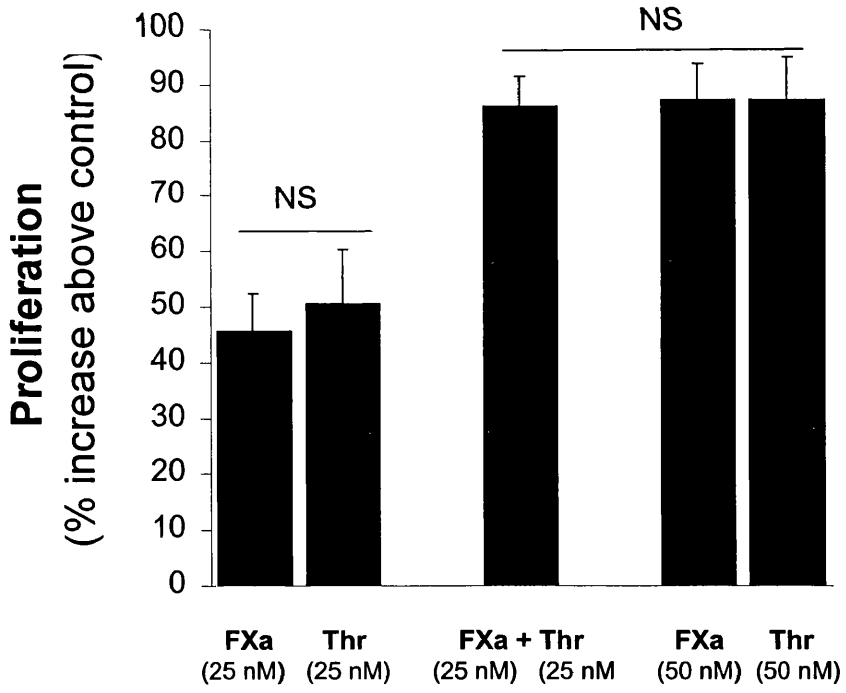


Figure 3.13 Factor Xa and thrombin have additional mitogenic effects on fibroblasts
Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., p values are calculated between the different treatments, mean of 2 experiments with 6 replicates for each value.

3.2.2.7 The Mitogenic Effect of Factor Xa is Mediated by the Stimulation of Platelet-derived Growth Factor

Figure 3.14 shows the mitogenic effect of fXa on human foetal lung fibroblasts in the presence and absence of a pan-specific polyclonal antibody (pAb) that neutralises the mitogenic effects of the three PDGF isoforms. FXa, thrombin and PDGF-AB stimulated fibroblast proliferation by 142±10%, 120±11% and 232±38% above medium control, respectively (all $p<0.01$). In contrast, fXa, thrombin and PDGF-AB did not stimulate proliferation significantly in the presence of PDGF neutralising antibody.

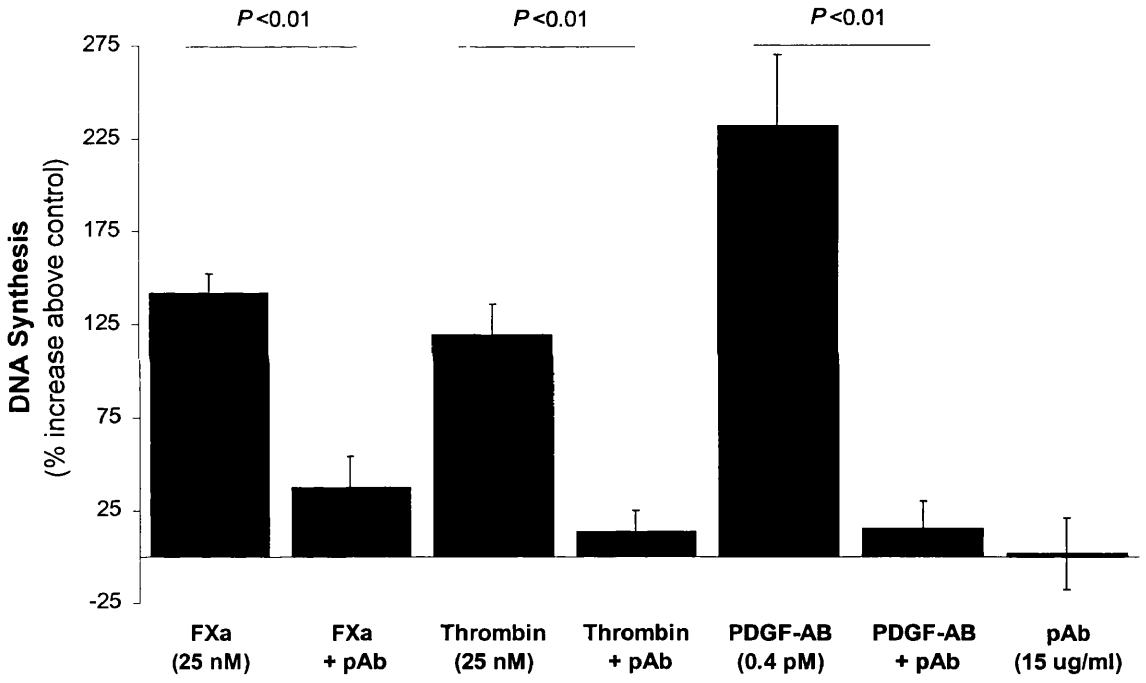


Figure 3.14 **An anti-PDGF antibody inhibits factor Xa-, thrombin- and PDGF-AB stimulated fibroblast DNA synthesis**

^{3}H-thymidine uptake assay; expressed in % increase above control ± s.e.m., p values are calculated between treatments, representative experiment with 6 replicates for each value.

3.3 DISCUSSION

The coagulation cascade serine proteases factors VIIa, IXa and Xa and thrombin are activated in the extrinsic coagulation pathway which is thought to be the primary haemostatic

mechanism brought to bear following tissue injury. Thrombin is also capable of regulating pleiotropic cellular functions such as activation-dependent gene transcription, autocrine mediator production, growth factor receptor expression and proliferation. It was hypothesised that other coagulation cascade proteases with structural features related to thrombin are mitogens for fibroblasts. The effects of factors VIIa, IXa and Xa on human foetal and adult fibroblast proliferation were characterised *in vitro*.

3.3.1 Effect of Coagulation Cascade Proteases on Fibroblast Proliferation

Factor Xa

FXa stimulates human foetal lung fibroblast DNA synthesis and proliferation *in vitro* in a dose-dependent manner between 1 and 200 nM. This range of active concentrations, the A_{max} of 100 nM and the amplitude of the effect (200% increase in proliferation above control) are very similar to those described for fXa in smooth muscle cells (Herbert *et al.*, 1998) and endothelial cells (Nicholson *et al.*, 1996). Thorough visual examination of the cells after 24 hr or 48 hr of incubation revealed no gross change in cell morphology or cell loss in either control or fXa-treated cells. Furthermore, the methylene blue dye binding assay and the direct cell counts are set up in such a way that there is no variation in cell number for up to 48 hr of incubation in wells treated with culture medium without additions. This excludes the possibility that fXa may only function as a survival factor, a function that thrombin can exert in human myobasts (Chinni *et al.*, 1999). Furthermore, the mitogenic effect of fXa is demonstrated by using three different experimental protocols, namely methylene blue dye binding assay, ^{3}H-thymidine incorporation assay and direct cell counts. Taken together, the results obtained with three independent assays confirm the validity of the finding that fXa is mitogenic for fibroblasts.

The maximal stimulatory concentration of fXa *in vitro* is below the normal human circulating concentration of the precursor factor X which approximates 140 nM (MacKie, 1996). Thus it is possible that concentrations of fXa may reach over 100 nM *in vivo*, in the vicinity or on the surface of cells at sites where the coagulation cascade is activated. Such concentrations of fXa might have proliferative effects on fibroblasts.

The proliferative effect of fXa is observed in human foetal lung fibroblasts, three types of primary human adult fibroblasts from normal lung, skin and heart, as well as SV40-transformed adult kidney fibroblasts. Thus, fXa appears to be mitogenic to fibroblasts from a broad variety of tissues. The mitogenic effect of fXa is more pronounced in human foetal lung fibroblasts. This

may be due to the higher basal proliferative potential of these cells which is also observed in response to other growth factors and serum (data not shown).

Two preparations from leading manufacturers, Calbiochem (La Jolla, CA, USA) and American Diagnostica (Greenwich, CT, USA), elicit similar proliferative responses in human foetal lung fibroblasts. This suggests that the mitogenic effect of fXa is not the eccentric characteristic of a single preparation. Finally, the specific thrombin inhibitor hirudin has no effect on fibroblast proliferation in response to fXa, suggesting that the mitogenic effect of fXa is not mediated by thrombin, whether produced locally by the fibroblasts, activated by fXa on the fibroblast surface or contaminating the fXa preparation. Furthermore, the neutralising mAb 5224 directed against the catalytic site of fXa markedly inhibited its' effect, confirming that the mitogenic effect observed is not due to contaminants within the fXa preparation. This is also the first evidence that the mitogenic effect of fXa requires the participation of its' catalytic site.

The finding that fXa is a potent mitogen for human fibroblasts contrasts with the previous report by Gasic and colleagues that fXa does not stimulate DNA synthesis in fibroblasts (Gasic *et al.*, 1992). However, this previous study had assessed the effect of human fXa on calf fibroblasts and transformed mouse fibroblasts. It is possible that these cells do not express the appropriate cell surface receptors necessary to the effects of human fXa, or that human fXa is unable to interact with bovine protease receptors. In the present study, all proteases and cells used are human. The mitogenic effect of fXa was observed in human foetal lung fibroblasts, human adult skin, heart and lung fibroblasts, SV40-transformed human adult kidney fibroblasts as well as mouse lung fibroblasts (section 4.2.3). Taken together, these data establish the potent mitogenic effect of this coagulation cascade protease on fibroblasts at near physiological concentrations.

Factor IXa

Factor IXa stimulates fibroblast DNA synthesis and proliferation significantly between 125 nM and 2000 nM. However, the circulating concentration of the precursor factor IX approximates 90 nM in normal human plasma (MacKie, 1996). It is possible that the high concentrations of factor IXa necessary to promote fibroblast replication (>125 nM) may not be achieved *in vivo*. Furthermore, thrombin or fXa could account for the entire stimulation observed if the factor IXa preparation employed held only about 0.3% of these proteases. In conclusion, the proliferative effect of factor IXa could potentially be attributed to a growth factor contaminating the protease preparation. To clarify the origin of the mitogenic activity observed, it would be of

interest to study the effect of factor IXa in the presence and absence of a specific factor IXa inhibitor or blocking antibody. Such specific reagents are not readily available.

Factor VIIa

Factor VIIa stimulates fibroblast DNA synthesis at concentrations between 25 nM and 200 nM. The maximal stimulation exhibited by factor VIIa at 100 nM is equivalent to that of fXa at the same concentration. This is the first time that the mitogenic effect of factor VIIa in mesenchymal cells is reported. The circulating concentration of the precursor factor VII is only 10 nM in normal human plasma (Mackie, 1996) and factor VIIa stimulates fibroblast proliferation significantly at concentrations superior to 25 nM, which is over two fold higher than the normal circulatory concentration of its' zymogen. It is unknown whether such concentrations of factor VIIa can be achieved *in vivo*, however, factor VIIa may contribute to the mitogenic effects of the coagulation cascade in conditions where of excessive and recurrent activation of the coagulation cascade.

Summary

There was a good correlation between the effect of the proteases on DNA synthesis as measured in the ^{3}H-thymidine uptake assay and their stimulation of proliferation measured with the methylene blue assay. These results corroborate and show that fXa is a potent mitogen for fibroblasts *in vitro* at near-physiological concentrations, whereas factor VIIa and factor IXa do not exhibit mitogenic activity at concentrations equivalent to that of their circulating zymogens. In addition, the maximal stimulatory concentration of fXa elicited an increase in proliferation comparable to that of a maximally stimulatory concentration of PDGF-AB (0.4 nM) or to about half of that of serum (10% NCS). These observations support the concept that coagulation cascade proteases other than thrombin stimulate fibroblast proliferation.

3.3.2 Mechanism of Action of Factor Xa -stimulated Fibroblast Proliferation

Factor Xa Stimulates Fibroblast Proliferation, but not its' Zymogen Factor X

Factor X promoted fibroblast proliferation in a dose-dependent manner between 10 nM and 100 nM. However, this stimulation was of modest amplitude and compared to only about 15% of that of fXa. The mitogenic effect of factor X could be due to a) factor X itself, b)

contamination of the factor X preparation by growth factors or proteases such as factor Xa or thrombin, c) the activation of fX into mitogenic concentrations of fXa on the surface of fibroblasts. Fibroblasts and other mesenchymal cells have been shown to express tissue factor constitutively and convert factor X into fXa with high efficiency. The incubation of fibroblasts in culture with factor X at a concentration of 200 nM for 1 hr generates a concentration of fXa of 25 nM (Brinkman *et al.*, 1994). This concentration of newly activated fXa would be mitogenic to fibroblasts and may account for the increased proliferation observed. It would be informative to try and block the mitogenic effect of fX with a specific inhibitor of factor X activation such as tissue factor pathway inhibitor (TFPI). Indeed, TFPI binds fXa as it is being activated by the factor VIIa/TF complex (Salemink *et al.*, 1998; for review, see Lindahl, 1997). However, purified TFPI was not readily available during the course of this thesis.

In summary, the proliferative activity of factor X was very modest in comparison with the effect of fXa and thrombin. This suggests that the activated state of the protease, including full proteolytic activity, is central to the its' proliferative function. To further characterise the mitogenic effect of fXa on fibroblasts, the role of the catalytic site of the protease was determined.

Role of the Catalytic Site of Factor Xa

To determine whether the catalytic site of fXa is necessary to its' proliferative effect on fibroblasts, three categories of inhibitors were employed. The naturally occurring anticoagulants TAP and ASN are fXa-specific catalytic site inhibitors produced respectively by the tick *Ornithodoros moubata* (Jordan *et al.*, 1992) and the Mexican leech *Haementeria offinalis* (Ohta *et al.*, 1994). Two molecules related to these natural inhibitors abrogate the proliferative effect of fXa completely. These inhibitors bind to the catalytic site of fXa in a reversible but highly specific fashion and compete with natural substrates. Complete inhibition is obtained with nanomolar concentrations of the inhibitors. However, high molecular ratios inhibitor/enzyme are necessary to obtain full inhibition over the 48 hr incubation of the proliferation assay (10:1 for rTAP and 16:1 for ASN related peptide). These inhibitors derived from natural molecules present the advantage of being active in complex physiological fluids. They have been efficiently used in a variety of studies as antithrombotic agents (Ragosta *et al.*, 1994; Wong *et al.*, 1996; Schwartz *et al.*, 1996) and provide an advantageous basis for the design of novel therapeutic peptide inhibitors of fXa. The neutralising effect of these inhibitors of fXa shows that the mitogenic activity of the protease is dependent on its' proteolytic activity. The small molecular weight compound DEGR-ck is a specific fXa-catalytic site inhibitor which binds covalently to the catalytic site and abrogates its'

activity irreversibly. Purified DEGR-fXa does not stimulate fibroblast proliferation. For this experiment, excess unbound inhibitor was removed from the inhibited fXa preparation by extensive dialysis. This rules out the possibility that free, unbound fXa inhibitor blocks fibroblast proliferation through non-specific mechanisms and confirms that the catalytic site of the protease is necessary to its' mitogenic effects. Finally, the fXa-neutralising mAb 5224 binds specifically to epitopes located to the catalytic pocket of fXa (Zacharski *et al.*, 1991) and thereby neutralises its' catalytic activity by steric hindrance and reversibly (Ko *et al.*, 1996). mAb 5224 completely inhibits the mitogenic effect of fXa. Collectively, these data demonstrate that the mitogenic effect of fXa on fibroblasts is mediated specifically by its' catalytic site. This is similar to the mode of action of thrombin on fibroblasts (Ohba *et al.*, 1994). The mitogenic effect of thrombin on fibroblasts is mediated by PDGF production and autocrine stimulation. In the next section, the possibility that fXa triggers a similar mechanism of growth factor production will be discussed.

PDGF Mediates the Mitogenic Effect of Factor Xa

A pan-specific PDGF-neutralising antibody inhibits the mitogenic effect of both fXa and thrombin nearly entirely. This suggests that the effect of fXa is entirely dependent on PDGF production and suggests that it acts as a secondary autocrine mediator. FXa has previously been shown to stimulate smooth muscle cell proliferation (Ko *et al.*, 1996) and endothelial cell proliferation (Gajdusek *et al.*, 1986; Nicholson *et al.*, 1996) via a similar PDGF-mediated mechanism. Furthermore, the stimulation of PDGF production is an characteristic similar to thrombin. Indeed, thrombin stimulates PDGF-AA production and proliferation in fibroblasts (Ohba *et al.*, 1994) and PDGF-AA production and proliferation in smooth muscle cells (Bydlowski *et al.*, 1998; Cucina *et al.*, 1999; Stouffer and Runge, 1998, for review). The total inhibition of the mitogenic effects of fXa with a blocking anti-PDGF antibody also suggests that this function is independent from the production of bFGF which has been reported in fibroblasts (Cucina *et al.*, 1999) and in endothelial cells (Joseph-Silverstein and Rifkin, 1987). Furthermore, the stimulation of fibroblast proliferation by fXa also appears to be independent from the action of interleukins such as IL-1 that fXa and thrombin stimulate in fibroblasts (Jones and Greczy, 1990), IL-6 that fXa and thrombin stimulate in smooth muscle cells and fibroblasts (Sower *et al.*, 1995; Papapetropoulos *et al.*, 1996), or IL-8 produced by endothelial cells in response to thrombin (Kaplanski *et al.*, 1997). This is similar to thrombin which stimulates fibroblast proliferation independently from its' effects on interleukin production (Ohba *et al.*, 1994; Loppnow *et al.*, 1998). Finally, the similarity between the potency and kinetics of fXa- and thrombin-stimulated

fibroblast proliferation suggests that the two proteases have similar consequences on PDGF production, secretion and autocrine stimulation in human fibroblasts.

In summary, the mitogenic effect of fXa is mediated via autocrine PDGF stimulation. This is consistent with previous observations in smooth muscle cells and comparable to the effect of thrombin in fibroblasts.

Factor Xa and Thrombin Have Similar Mitogenic Effects

The characteristics of the mitogenic effects of fXa and thrombin were compared using three different methods to assess cellular proliferation: The ^{3}H-thymidine incorporation assay, the methylene blue colorimetric assay and direct cell counts. Firstly, the concentration range over which fXa and thrombin exhibit proliferative activity are strictly similar (1 nM to 200 nM), as well as their maximally stimulatory concentrations (100 nM) in all the assays used. This data shows that fXa and thrombin are equally potent to stimulate fibroblast proliferation. A similar observation has already been made in endothelial cells (Nicholson *et al.*, 1996) and in smooth muscle cells (Herbert *et al.*, 1998).

Second, fXa upregulated fibroblast proliferation with a delayed onset typical of thrombin (Molloy *et al.*, 1996) and there was no statistical difference between stimulation of fXa (25 nM) or thrombin (25 nM) at any time point over 72 hr of incubation in the methylene blue assay. This delay has been attributed to the production of secondary mediators with autocrine mitogenic activity (Molloy *et al.*, 1996). To further confirm this hypothesis, the mitogenic activity of fXa and thrombin were both blocked completely with a neutralising antibody directed against PDGF suggesting that the effects of the proteases are mediated by the same growth factor.

Third, the catalytic activity of fXa is critical to its' mitogenic effects, similar to thrombin, and the inactive precursor (Factor X) or catalytically-inhibited forms of the protease show no significant mitogenic potential.

Finally, the stimulation of fibroblast proliferation by fXa and thrombin are exactly additive and not synergistic when added to the cells in combination. Yet, fibroblasts in these experiments had the potential to proliferate above these levels since serum (10% NCS) solicited a much greater increase in proliferation.

These observations suggest that fXa and thrombin stimulate fibroblast proliferation via similar molecular mechanisms that include secondary growth factor stimulation and may involve shared cell surface receptors.

Summary

The mitogenic effect of factors VIIa, IXa and fXa have been assessed on human fibroblasts and compared to that of thrombin. Three types of assays revealed that fXa and thrombin are the most potent mitogens out of the extrinsic coagulation pathway proteases. FXa has a similar effect to thrombin and stimulates both foetal and adult human fibroblast proliferation. A selection fXa-catalytic site inhibitors and neutralising antibodies acting through different mechanisms, including substrate competition, covalent binding and steric hindrance, inhibit the mitogenic activity of fXa, suggesting that the catalytic activity of the protease mediates its' mitogenic effect. In addition, the factor X circulating precursor had little effects, demonstrating that the enzyme acquires its' mitogenic potential when its' is activated. Furthermore, the mitogenic effect of fXa on fibroblasts was mediated by the autocrine stimulation of PDGF, similar to its' effects in smooth muscle cells and to that of thrombin on fibroblasts and smooth muscle cells. Taken together, these data suggest that coagulation fXa is a potent mitogen for fibroblasts. In the next chapter, I will begin to unravel the cell surface receptor system that mediates the mitogenic effect of fXa on fibroblasts.

CHAPTER 4

EPR-1 AND PAR-1 MEDIATE THE MITOGENIC EFFECTS OF FACTOR Xa ON FIBROBLASTS

In chapter 3, the mitogenic potential of the extrinsic coagulation pathway proteases was characterised in human fibroblasts. FXa was shown to have mitogenic effects similar to thrombin for these cells. To further characterise this novel function of fXa, the present chapter will focus on the molecular mechanism through which fXa stimulates fibroblast proliferation. Effector cell protease receptor-1 (EPR-1) is a novel cell surface receptor for fXa that has been described in leukocytes and smooth muscle cells (Altieri and Edgington, 1990; Herbert *et al.*, 1998). The first part of this chapter aims to determine the expression and role of EPR-1 in the mitogenic effect of fXa on fibroblasts. The second objective is to investigate the idea that a second protease receptor acts in conjunction with EPR-1 to mediate the effect of fXa on fibroblasts. To address these aims, PAR activation by fXa will be studied by measuring cytosolic Ca^{2+} mobilisation in human fibroblasts and genetically engineered PAR-1-deficient mouse fibroblasts. In addition, the role of intracellular Ca^{2+} signalling in response to fXa will be studied.

4.1 INTRODUCTION

4.1.1 EFFECTOR CELL PROTEASE RECEPTOR-1

It is commonly assumed that fXa interacts with negatively charged phospholipids to bind to the surface of platelets and other cells (chapter 1). However, a protease receptor has been implicated as the high affinity binding site for fXa in monocytic cells and lymphocytes (Altieri and Edgington, 1989; Altieri and Edgington, 1990) and other cells. This receptor was first termed *cellular factor Va* due to its' analogy to the fXa cofactor, factor Va (Altieri and Edgington, 1989). It was later renamed effector cell protease receptor-1 (EPR-1). EPR-1 contributes to the assembly of the prothrombinase complex during blood coagulation by binding fXa and localising the protease to procoagulant cell surfaces, as summarised in the general introduction of this thesis. In

the next paragraphs, the activation mechanism and cellular events linked to EPR-1 will be reviewed.

4.1.1.1 Effector Cell Protease Receptor-1 Ligation

EPR-1 is an unusually basic glycoprotein of 337 amino acids (36.8 kDa M_r; Altieri, 1994a). It contains a single putative 81 amino acid transmembrane domain (Altieri *et al.*, 1994a), a short, serine-rich cytoplasmic tail and an extracellular domain ending with a short hydrophobic domain (Ambrosini and Altieri, 1996). Two regions of EPR-1 interact with fXa (Ambrosini and Altieri, 1996). One is involved in factor Va-independent prothrombinase assembly (residues Pro^{120}-Ala^{154}) and one mediates lymphocyte activation by fXa (residues Met^{1}-Arg^{60}). The first interaction occurs between residues Pro^{120}-Ala^{154} of EPR-1 and residues Leu^{83}-Leu^{88} of fXa. This region of fXa is located between the two EGF repeats of the light chain (Ambrosini and Altieri, 1996) and it is only made available to the receptor after activation of the enzyme (Ambrosini *et al.*, 1997). Little is known about the significance of these interactions or the intracellular signalling events that they lead to. Nevertheless, it has been established that EPR-1 is not cleaved or proteolytically activated by fXa (Altieri and Edgington, 1990).

4.1.1.2 Cellular Effects Mediated by EPR-1

In addition to promoting factor Va-independent prothrombinase assembly, there is increasing evidence that this EPR-1 also plays a central role in mediating cellular effects of fXa, such as smooth muscle cell proliferation (Herbert *et al.*, 1998) and inflammatory cytokine production (Cirino *et al.*, 1997) *in vitro* and *in vivo*. For instance, short EPR-1 antagonist peptides inhibit interleukin production and other pro-inflammatory effects of fXa *in vivo* (Papapetropoulos *et al.*, 1998). In addition, binding of fXa to EPR-1 is necessary to cause the release of PDGF and autocrine stimulation of smooth muscle cells (Herbert *et al.*, 1998; Kho *et al.*, 1996). EPR-1 also mediates lymphocyte activation in response to fXa (Altieri and Stamnes, 1994) and EPR-1 antagonists inhibit the stimulatory effects of fXa on antibody and IL-2 production by lymphocytes *in vivo* (Duchosal *et al.*, 1996). Thus EPR-1 ligation mediates most of the known cellular effects of fXa. In addition, the catalytic activity of fXa has also been reported to play a role in the mitogenic effect of fXa as specific inhibitors such as rTAP abolish its' mitogenic effect in smooth muscle cells (Gasic *et al.*, 1992; Kho *et al.*, 1996) and endothelial cells (Nicholson *et al.*, 1996).

However, EPR-1 does not appear to be cleaved by fXa (Altieri and Edgington, 1990) and the role of the proteolytic activity of fXa remains unclear (Ko *et al.*, 1996; Herbert *et al.*, 1998).

4.1.1.3 Signalling Pathways

EPR-1 only has a short cytoplasmic tail which contains potential phosphorylation residues, but it has not yet been clearly associated with either binding of membrane-bound signalling proteins or particular phosphorylation patterns (Altieri, 1994a). In consequence, the signalling events that have been described in response to fXa such as cytosolic Ca^{2+} mobilisation in endothelial cells (Nicholson *et al.*, 1996) and smooth muscle cells (Herbert *et al.*, 1998) can not be attributed to EPR-1 activation with certainty. There is additional evidence that the stimulation of smooth muscle cells with fXa triggers activation of the mitogen-activated protein kinase (MAPK) signalling cascade, including the secondary mediators Ras, Raf, p42MAPK and p44MAPK, as well as the accumulation of the transcription factors c-Jun and c-Fos (Ko *et al.*, 1996). However, EPR-1 has not been formerly involved in these events. In addition, there are discrepancies between reports on the secondary mediators stimulated by fXa. In one publication, Ko and colleagues observed that fXa does not trigger inositol 1,4,5-trisphosphate formation or cytosolic Ca^{2+} mobilisation in smooth muscle cells (Ko *et al.*, 1996). Since then, Herbert and colleagues have reported an accumulation of inositol 4-phosphate in smooth muscle cells in response to fXa (Herbert *et al.*, 1998). This may reflect a stimulation of inositol 1,4,5-trisphosphate metabolism and possibly phospholipase C activity. Furthermore, it is known that human fXa does indeed induce cytosolic Ca^{2+} mobilisation in Madin-Darby canine kidney cells (Camerer *et al.*, 1996) and *Xenopus laevis* oocytes transfected with a human PAR-1 construct (Ishihara *et al.*, 1997).

In summary, binding to EPR-1 is necessary for the cellular effects of fXa, but discrete signalling events have not been formerly associated with EPR-1 ligation and the role of cytosolic Ca^{2+} mobilisation in response to factor Xa has not been elucidated.

4.1.1.4 Is there a Second Receptor for Factor Xa?

The role of EPR-1 in the cellular effects of fXa has been reviewed in the previous sections. However, the catalytic site of fXa is also involved in the cellular effects of fXa and it is clear that several proteins form a complex system that transduces the cellular effects of fXa. It has been postulated that protease-activated receptors may be implicated in the mitogenic effects of

fXa. I*n vitro* assays gave some biochemical evidence that fXa in solution can cleave synthetic peptides containing the activation sites of PAR-1 (Parry *et al.*, 1996) or PAR-2 (Fox *et al.*, 1997). Other groups reported that fXa had no effect on synthetic peptides mimicking the PAR-2 amino terminus (Molino *et al.*, 1997b). In contrast, fXa can cleave PAR-1 mimic peptides with a specificity constant 2.10^5 times lower than that of thrombin (Parry *et al.*, 1996). This suggests that in the absence of cofactors or membrane phospholipids, fXa is a poor activator of PAR-1 compared with thrombin. Nevertheless, these experiments demonstrate on a biochemical basis that fXa cleaves PAR-1 at the activation site and that a close molecular interaction between fXa and the PAR-1 amino terminus is possible. PAR-1 and PAR-2 were shown to transduce the mitogenic signals of proteases such as thrombin and trypsin in smooth muscle cells (section I.1.3). The activation of PAR-1 by thrombin induces the release of autocrine PDGF which stimulates cell replication (Ohba *et al.*, 1996; Bono *et al.*, 1997b). Thus it seems plausible that PAR-1 may mediate the release of PDGF in response to fXa. In summary, EPR-1 ligation is involved in most cellular effects of fXa. However, the receptor mechanism for fXa remains largely undefined and it is possible that PAR-1 or PAR-2 mediate the effects of the protease.

4.1.2 PROTEASE-ACTIVATED RECEPTORS

A protease-activated receptor, PAR-1, mediates the majority of the cellular effects of thrombin on fibroblasts. Thus PAR-1 or other members of the protease-activated receptor family may mediate the effects of coagulation cascade serine proteases such as fXa. To investigate if a protease-activated receptor is stimulated by fXa and mediates its' mitogenic effects, it is important to understand how such receptors are activated and the intracellular consequences of their activation. PAR-1 is the most studied PAR and it serves as a model to understand the biology of these receptors. The biochemistry of PAR-1 activation, its' signalling pathways and desensitisation will be discussed below. The recent findings about the biology of PAR-2, PAR-3 and PAR-4 are also introduced briefly.

4.1.2.1 PAR-1 Activation

Activation by Thrombin

The *thrombin receptor* PAR-1, is a seven transmembrane domain G-protein-coupled receptor of about 425 amino acids. The M_r of the mature receptor approximates 36 to 40 kDa (Vu *et al.*, 1991).

Over the last 8 years, the existence of more than one PAR-1 agonist has been suggested (Ofosu *et al.*, 1998) but thrombin is still regarded as the only physiologically relevant agonist of PAR-1. The activation of PAR-1 by thrombin is representative of the unique mechanism of PAR stimulation.

PAR-1, like all protease activated receptors, is a G protein-coupled receptor with seven transmembrane domains (reviewed in Ji *et al.*, 1998). Thrombin activates PAR-1 in a unique manner involving limited proteolysis rather than ligand binding only (Hung *et al.*, 1992a). Thrombin cleaves a 41 amino acid segment of amino-terminal end of the receptor ($LDPR^{41}\downarrow S^{42}FLLRN$), revealing a new 33 residue amino terminus. The terminal sequence $S^{42}FLLRNPNDKYEPF^{55}$ functions as a tethered ligand and binds intramolecularly to the body of the receptor (Nanevicz *et al.*, 1996 and diagram 6 below).

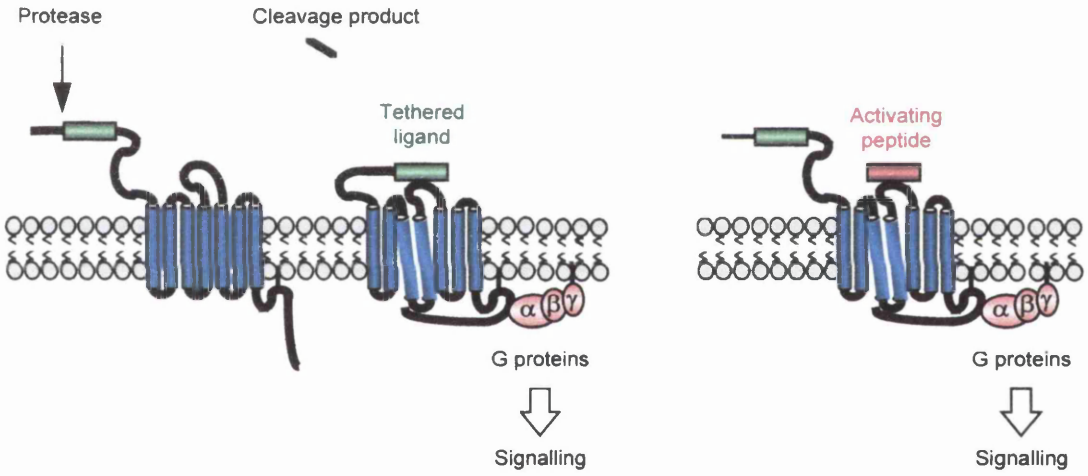


Diagram 6 **Mechanism of protease-activated receptor stimulation by endoproteolysis and activating peptides**

This diagram shows the main mechanisms of PAR activation. These receptors are activated mainly by an irreversible step of *limited endoproteolysis* catalysed by serine proteases *in vivo*. Proteolysis of the receptor in its' extracellular domain reveals a new amino terminus which binds to discrete regions of the receptor and triggers conformational changes, heterotrimeric G protein coupling and signalling. This amino terminus is termed *tethered ligand* (left panel). However, PAR activation can be reproduced by short synthetic peptides mimicking the natural sequence of tethered ligands. These *activating peptides* bind to the same extracellular regions of the receptor recognised by the natural tethered ligand, but in a reversible fashion (right panel).

Amino acid substitutions in the extracellular face of PAR-1 with segments corresponding to the PAR-2 extracellular loops yielded chimeras with PAR-2-like selectivity for agonists (Lerner *et al.*, 1996) implying that these regions play a role in ligand recognition.

The second of the three extracellular loops of the receptor seems to be critical to this binding process and determines the agonist specificity of the receptor (Lerner *et al.*, 1996). Binding of the tethered ligand induces a change in the conformation of the receptor (reviewed in Gether and Kobilka, 1998). Although the precise nature of this modification is not understood, this conformational change in the activated receptor allows it to interact with heterotrimeric G proteins in the plasma membrane which in turn transduce the intracellular signals (Crouch and Simon, 1997).

Synthetic Activating Peptides

Synthetic peptides corresponding to the first 5 to 14 amino acids of the tethered ligand of PAR-1 agonise the receptor through binding to the extracellular *face* of the receptor, formed by the extracellular loops (diagram 5). These synthetic agonist peptides, including SFLLR and SFLLRNP (Garcia *et al.*, 1993), are based on the human PAR-1 sequence and have varying degrees of selectivity for either PAR-1 or PAR-2 (Cheung *et al.*, 1998). Studies investigating the specificity of these PAR-1 activating peptides have shown that cells expressing PAR-2 respond to both PAR-1 and PAR-2 activating peptides (Lerner *et al.*, 1996; Kawabata *et al.*, 1999). To increase the specificity of PAR-1-activating peptides, the amino terminal serine residue was substituted for a threonine. This was inspired by the *Xenopus laevis* PAR-1 amino terminal sequence (TFRIFD) and lead to the creation of TFLLR, an agonist with superior selectivity for PAR-1 (Darrow *et el.*, 1996; Hollenberg *et al.*, 1997).

4.1.2.2 PAR-1 and Thrombin Signalling Pathways

The cellular consequences of PAR-1 activation by thrombin have been examined in endothelial cells and fibroblasts. This provides us with a thorough insight on the signalling pathways linked to this receptor. In particular, PAR-1, PAR-2, PAR-3 and PAR-4 activation are coupled to the immediate mobilisation of cytosolic free Ca^{2+} (Déry *et al.*, 1998, for review). Thus Ca^{2+} measurements can be used to assess the stimulation of these receptors. In the next sections, a particular focus will be brought onto the regulation of cytosolic free Ca^{2+} by PAR-1. The

transduction of intracellular signals by PAR-1 is effected through interactions with heterotrimeric G proteins that contain a Gα, Gβ or Gγ subunit (Hamm, 1998 for review).

At least two different pathways appear to be stimulated following PAR-1 activation and involve different Gα subtypes, including Gαi, Gαq or possibly Gαo proteins (Crouch and Simon, 1997 and diagram 7). These Gα subunits are likely to be coupled to the receptor by its' second cytoplasmic loop (Verrall *et al.*, 1997). Activation of these G proteins leads to Ca^{2+} mobilisation and the activation of the 42 kDa and 44 kDa mitogen-activated protein kinases, or p42MAPK and p44MAPK (Gutkind, 1998 for review). In turn, these protein kinases are translocated to the nucleus where they phosphorylate transcription factors, promote gene transcription and proliferation (Baffy *et al.*, 1994; Trejo *et al.*, 1996).

The $G\alpha_q$ Protein Pathway

In fibroblasts and endothelial cells, PAR-1 activation leads to the activation of inositol-phosphate-specific phospholipase C in a Gαq protein-dependent manner (summarised in diagram 5). In turn, phospholipase C catalyses the breakdown of phosphatidylinositol 4,5-bisphosphate (Garcia *et al.*, 1991). This leads to the production of the Ca^{2+} secretagogue inositol 1,4,5-trisphosphate and the accumulation of 1,2-diacylglycerol (Garcia *et al.*, 1993). Subsequently, inositol 1,4,5-trisphosphate stimulates specific Ca^{2+} channels on the endoplasmic reticulum membrane and provides the main pathway to cytosolic Ca^{2+} mobilisation (Quinton *et al.*, 1996). Thrombin and synthetic PAR-1 activating peptides induce cytosolic Ca^{2+} mobilisation. This appears to be mediated by the upregulation of inositol 1,4,5-trisphosphate formation in fibroblasts (Baffy *et al.*, 1994), myofibroblasts (Mathias *et al.*, 1998), smooth muscle cells (Kaufmann *et al.*, 1997), cardiac myocytes (Jiang *et al.*, 1998), endothelial cells (Garcia *et al.*, 1993), megakaryocytes (Jones *et al.*, 1992) and Madin-Darby canine kidney cells (Camerer *et al.*, 1996).

Cytosolic Ca^{2+} mobilisation from endogenous stores occurs within 10 sec of stimulation with thrombin or synthetic PAR-1 activating peptides (Garcia *et al.*, 1993). The effect of thrombin on intracellular Ca^{2+} is mainly Pertussis toxin-independent, suggesting a Gαq protein-mediated mechanism (Garcia *et al.*, 1991; Garcia *et al.*, 1992b). However, cytosolic Ca^{2+} mobilisation is also partly Cholera toxin-independent (Patterson *et al.*, 1994), suggesting that several pathways link PAR-1 to Ca^{2+} signalling (Garcia, 1992a, for review).

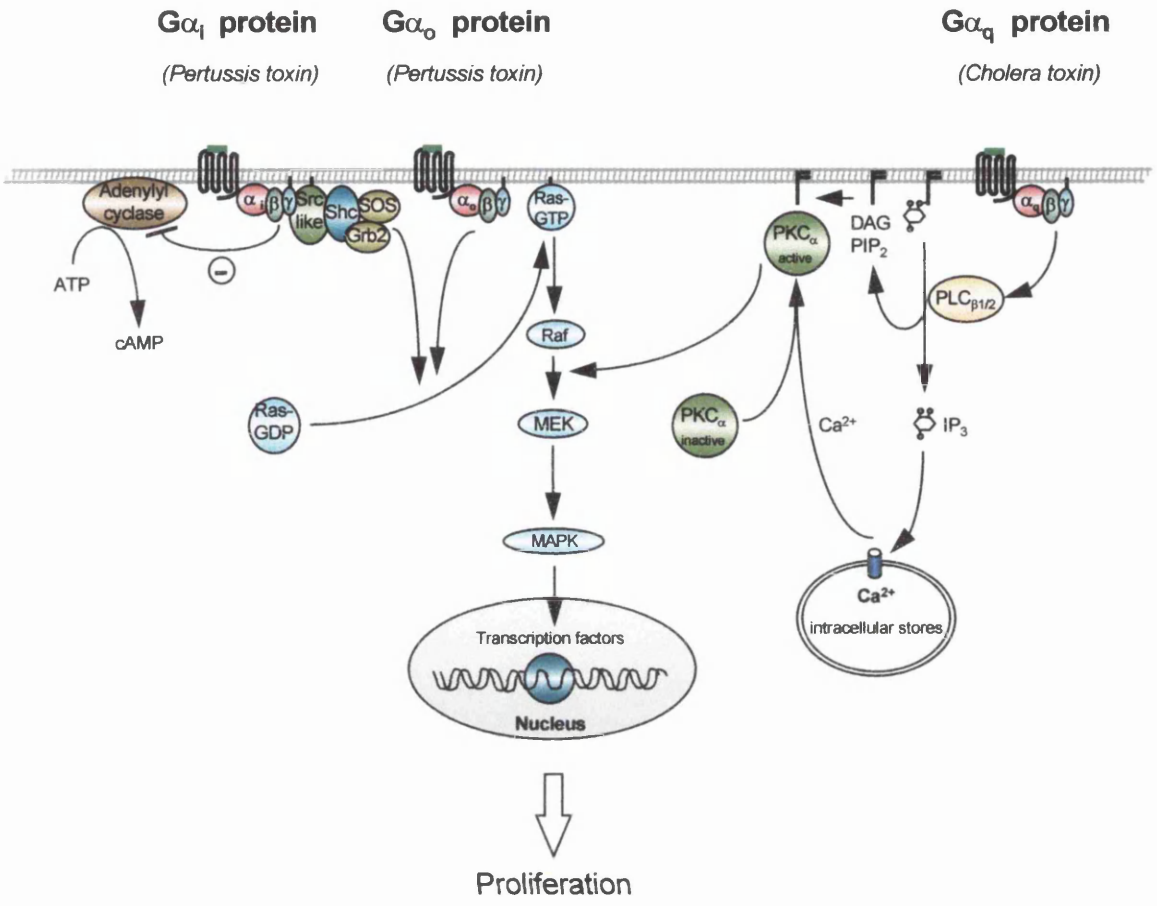


Diagram 7 **PAR-1 signalling cascades in fibroblasts**

This diagram shows the main PAR-1 signalling cascades that lead to cytosolic Ca^{2+} mobilisation and cell proliferation. The different G proteins coupled to PAR-1 are identified along their inhibitors (*in italics*).

- The Gαi pathway involves activation of Src and complex formation with the adapter protein Shc, Grb2 and the Ras activator SOS which in turn stimulates Ras, Raf and the MAPK cascade. Moreover, Gαi G proteins inhibit adenylyl cyclase activity and the formation of cyclic 3':5'-adenosine monophosphate (cAMP) from adenosine triphosphate (ATP). This inhibition potentiates Ca^{2+} mobilisation.
- The Gαq pathway involves activation of phospholipase $C_{\beta 1}$ and $_{\beta 2}$ (PLC $_{\beta 1/ 2}$) that hydrolyse phosphoinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and 1,2-diacylglycerol (DAG). This leads to activation of protein kinase C (PKC), MAPK kinases (MEK) and mitogen-activated protein kinases (MAPK).

The Gα$_i$ Protein Pathway

The tyrosine kinase protein kinase C plays a role in PAR-1 signalling via Gαq proteins (Trejo *et al.*, 1996 and cytosolic free Ca^{2+} confers their activity to several protein kinase C isoforms (Ha and Exton, 1993). Active protein kinase C activates Raf which feeds directly into the MAPK cascade (Soh *et al.*, 1999) and leads to fibroblast proliferation (Molloy *et al.*, 1996). Thus protein kinase C stimulates the MAPK cascade at several levels to promote cell proliferation. Gαq protein coupling to PAR-1 also stimulates the activation of membrane-bound phospholipases (Garcia *et al.*, 1991) as some of these enzymes depend on intracellular Ca^{2+} mobilisation to gain their full activity. In summary, several partly defined pathways link PAR-1 to cytosolic Ca^{2+} mobilisation and proliferation.

The second main PAR-1 signalling pathway is Gαi protein-dependent and possibly c-Raf-independent (Trejo *et al.*, 1996; For review, see Lefkowitz, 1998). This pathway (summarised in diagram 6) may also lead to cell proliferation by inducing intracellular tyrosine kinase phosphorylation, but it does not appear to be directly linked to Ca^{2+} mobilisation. In fibroblasts, activation of PAR-1 stimulates Src tyrosine kinase phosphorylation in a mechanism that is partially sensitive to Pertussis toxin, suggesting that Gαi proteins are involved in this process (Chen *et al.*, 1994). In addition, Gαo proteins may also associate with PAR-1 and stimulate Ras and the MAPK signalling cascade in a similar way to Gαi proteins (Hung *et al.*, 1992b). Following PAR-1 activation, the tyrosine kinase Src phosphorylates Ras via the tyrosine phosphoprotein adapter Shc. Shc is recruited and complexed with Grb2 and the Ras exchange factor SOS in a classical mechanism (Chen *et al.*, 1996) and Src mediates the activation of p42MAPK and p44MAPK in response to thrombin, leading to proliferation (Luttrell *et al.*, 1997; Della Rocca *et al.*, 1999). There are alternative scenarios that may link Gαi protein activation to proliferation. It has also been suggested that Src may in fact interact with Gβγ protein heterodimers independently from the Gα isoform originally involved in coupling with the receptor and Gβγ heterodimers may be able to directly activate Shc (van Biesen *et al.*, 1995), relay receptor. Coupling of Gαi proteins to PAR-1 also has the effect of inhibiting adenylyl cyclase activity, thereby down-regulating cyclic 3':5'-adenosine monophosphate accumulation (Hung *et al.*, 1992b) which inhibits inositol 1,4,5-trisphosphate-gated Ca^{2+} channels on the endoplasmic reticulum membrane. This effect is thought to potentiate Ca^{2+} mobilisation in response to thrombin and it can be reversed using Cholera toxin (Garcia *et al.*, 1991; Garcia *et al.*, 1992b). Finally Gαi proteins also stimulate pospholipase A_2 which mediates prostaglandin E2 formation

by fibroblasts (Derian and Eckardt, 1997). However, these events occur mostly in a Ca^{2+}-independent fashion and are not directly connected to proliferation (Winitz *et al.*, 1994).

The Mitogen-Activated Protein Kinase Pathway

Thrombin stimulation of PAR-1 upregulates the activation of the MAPK signalling cascade in vascular smooth muscle cells (Molloy *et al.*, 1996) and fibroblasts (Trejo *et al.*, 1996) and involves the phosphorylation of p42MAPK and p44MAPK (Trejo *et al.*, 1996; reviewed in Lefkowitz, 1998). p42MAPK and p44MAPK are necessary and sufficient for fibroblast proliferation (reviewed in Pages *et al.*, 1993). In contrast, there is practically no stimulation of p38MAPK by PAR-1 agonists in fibroblasts (Belham *et al.*, 1996).

Summary

In this section, the diverse G protein-dependent signalling pathways triggered by PAR-1 activation have been described. Within seconds, the activation of PAR-1 leads to cytosolic Ca^{2+} mobilisation . This is mediated at least in part by association of PAR-1 with Gαq proteins and phospholipase C activity, but it is also potentiated by coupling of PAR-1 with Gαi proteins and the inhibition of adenylyl cyclase. Cytosolic free Ca^{2+} plays a role in the transduction of the mitogenic signal from PAR-1 to the nucleus by promoting the activity of Ca^{2+}-dependent kinases such as protein kinase C. Due to the irreversible nature of receptor activation by proteolysis, strong regulatory mechanisms are required to attenuate the signal after PAR-1 stimulation. These mechanisms are discussed in the next section.

4.1.2.3 PAR-1 Inactivation and Desensitisation

The mechanism of receptor activation by limited proteolysis and tethered ligand binding has unique characteristics. These are mainly due to the irreversible nature of PAR activation by proteases. First, activated receptors become resistant to further proteolytic activation. Second, the rapid cleavage of protease-activated receptors on the cell surface renders cells unresponsive to further stimulation. This remains true until new receptors are expressed on the cell surface, which occurs through the release of intact receptors from intracellular stores or through *de novo* synthesis (Déry *et al.*, 1998, for review). Finally, activated receptors are locked in a signalling conformation due to the fact that their ligands remain attached to them (Hammes and Coughlin, 1999). As a consequence, the attenuation of protease-activated receptor signalling cannot occur through the

removal of the agonist by diffusion and requires specific inactivation mechanisms. The attenuation of protease-activated receptor signalling can occur through extracellular proteolytic *deactivation* of the receptor by proteases such as trypsin (Hammes and Coughlin, 1999), neutrophil elastase, cathepsin G (Renesto *et al.*, 1997), tryptase and chymase from mast cells (Schechter *et al.*, 1998) and finally plasmin (Kuliopulos *et al.*, 1999). These proteases can cleave and remove the tethered ligand of the PAR-1 amino terminus, thereby terminating the signal (reviewed in Déry *et al.*, 1998).

The attenuation of protease-activated receptor signalling can also occur intracellularly through disarming of the receptors in a process termed *desensitisation*. In this scenario, the receptor is actively incapacitated by the cell through the phosphorylation of specific sites that prevent coupling with G proteins (Déry *et al.*, 1998). Phosphorylation of PAR-1 and PAR-2 is initiated by a specific class of G protein receptor kinases that function with cofactors or *arrestins* (Bunemann and Hosey, 1999 for review). It has been suggested very recently that arrestins are endocytosed with the activated protease-activated receptor into early endosomes and then separate from it when the receptor is directed to lysosomes (Déry *et al.*, 1999). There is also evidence that PAR-1 desensitisation may be achieved via second messenger kinases such as protein kinase C (Ishii *et al.*, 1994; Yan *et al.*, 1998).

4.1.2.4 PAR-1 Endocytosis and Trafficking

In the absence of agonist, PAR-1 shuttles continuously between the cell surface and the intracellular stores (Shapiro *et al.*, 1996). Fibroblasts and other cells possess large intracellular pools of PAR-1 and PAR-2 (Hein *et al.*, 1994). The greater part of these PAR-1 stores is located in the endoplasmic reticulum (Horvat and Palade, 1995). However, activation of PAR-1 has a rapid effect on the cell surface expression of the receptor. As shown in megakaryocytes and fibroblasts, over 98% of cell surface PAR-1 are activated and endocytosed after only two minutes of incubation with thrombin at physiological concentrations (Hein *et al.*, 1994; Brass *et al.*, 1994). Initially, activated PAR-1 are accumulated into coated pits in the cell membrane in a cadherin-dependent mechanism. Subsequently, activated PAR-1 are directed towards endosomes (Hoxie *et al.*, 1993). Within 30 min to 60 min they traverse the endosomal network and are sorted to lysosomes (Hoxie *et al.*, 1993; Hein *et al.*, 1994). The cytoplasmic tail of the receptor plays a critical role in this process (Shapiro *et al.*, 1996; Trejo and Coughlin, 1999). However, a portion of activated PAR-1 escapes the sorting process and finds itself re-expressed on the cell surface

(Shapiro *et al.*, 1996; Ellis *et al.*, 1999b). These receptors cannot be activated again and their function remains unknown (Hoxie *et al.*, 1993). Recent observations have implied that the cleaved receptors that are recycled to the cell surface may in fact still be able to transduce signals in response to alternative agonists, such as synthetic activating peptides (Hammes and Coughlin, 1999). This provides an alternative mechanism for the attenuation of PAR stimulation.

4.1.2.5 Other Protease-Activated Receptors

More recently, three extra members of the PAR family have been discovered and termed PAR-2, -3 and -4. First, PAR-1, PAR-2 and PAR-3 are located in the same region of the genome (chromosome 5q13) and share high levels of homology (Schmidt *et al.*, 1996; Nystedt *et al.*, 1995; Guyonnet-Duperat *et al.*, 98). At variance, PAR-4 is located on chromosome 19p12 (Xu *et al.*, 1998). It has been hypothesised that these receptors originated from a single ancestor by gene duplication and transposition (Kahn *et al.*, 1996). It may be that several more protease-activated receptors arose from such gene duplication events and remain to be discovered. PAR-3 and PAR-4 are endoproteolytically activated by thrombin similarly to PAR-1. However, PAR-2 is activated by a different class of serine proteases related to trypsin (Molino *et al.*, 1997b). All of these receptors are potential mediators of the mitogenic effect of fXa. Relevant information on their biology will be discussed briefly.

PAR-2

PAR-2 is a 397 amino acid seven transmembrane domain receptor with about 35% similarity with PAR-1 at the protein level (Nystedt *et al.*, 1995; Kahn *et al.*, 1996). To date, PAR-1 and PAR-2 are the only protease-activated receptors described in fibroblasts. PAR-2 is proteolytically activated (table 5) and mediates the cellular effects of serine proteases related trypsin (Molino *et al.*, 1997b; Molino *et al.*, 1997c) and mast cell tryptase (Schechter *et al.*, 1998). In contrast, PAR-2 is not activated by thrombin (Fox *et al.*, 1997). Similarly to PAR-1, PAR-2 activation has been implicated in the stimulation of the MAPK signalling cascade (Belham *et al.*, 1996) as well as cell growth in response to trypsin and mast cell tryptase in fibroblasts (Ruoss *et al.*, 1991; Belham *et al.*, 1996; Mirza *et al.*, 1997;) and smooth muscle cells (Bono *et al.*, 1997b; Bretschneider *et al.*, 1999).

Receptor	Region	Protease cleavage site	Agonist
PAR-1	residues 37 to 61	TLDPR ▼**SFLLRNP**NDKYEPFWEDEEK	**Thrombin**
PAR-2	residues 32 to 56	SSKGR ▼**SLIGKV**DGTSHVTGKGVTVE	**Trypsin, tryptase**
PAR-3	residues 34 to 57	TLPIK ▼ TFRGAPPNSFEEFPFSALE	**Thrombin**
PAR-4	residues 28 to 52	LPAPR ▼**GYPGQV**CANDSDTLELPDSS	**Thrombin**

Table 5 **Protease cleavage sites in PAR-1, PAR-2, PAR-3 and PAR-4**
The protease cleavage sites within the amino terminus of each human protease-activated receptor known to date is represented (arrow). Residues in bold are thought to form the tethered ligand region of PAR-1, PAR-2 and PAR-4. The information on a putative PAR-3 tethered ligand remains unclear.

PAR-3

PAR-3 was the second thrombin receptor to be discovered. It is over 300 amino acid long and shares about 28% similarity with PAR-1 (Cupit, 1999, for review). Although PAR-3 is proteolytically activated by thrombin (table 5), there is little information on a putative tethered ligand region in the receptor and no specific activating peptide has been described to date. The tissue distribution of PAR-3 in humans remains largely unexplored, but the receptor has been found in megakaryocytes and in the bone marrow, in the heart and in the liver (Ishihara *et al.*, 1997). PAR-3 has also been identified in mouse platelets and splenic megakaryocytes (Schmidt *et al.*, 1998 and section 3.1). In human platelets, PAR-3 mediates inositol 1,4,5-trisphosphate metabolism and may be linked to Ca^{2+} mobilisation (Ishihara *et al.*, 1997; Quinton *et al.*, 1996). Because the PAR-3 gene is within 80 kb only downstream from that of PAR-1, it has been postulated that the these genes may share regulatory elements and have potentially co-ordinated expression patterns (Kahn *et al.*, 1998a).

PAR-4

PAR-4 is the most recent receptor of the protease-activated family to be cloned (Xu *et al.*, 1998). PAR-4 is about 385 amino acid long and shows about 33% homology to PAR-1, PAR-2 and PAR-3. The physiological role of PAR-4 has been mainly associated with platelet aggregation in humans (section 3.1). PAR-4 is proteolytically activated by thrombin and short synthetic peptides mimicking the amino terminus of this receptor can reproduce the effects of thrombin on this receptor (Kahn *et al.*, 1998b) which implies a very similar mechanism of activation to that of

PAR-1 and PAR-2. Furthermore, activation of PAR-4 by thrombin and activation peptides leads to inositol 1,4,5-trisphosphate metabolism and cytosolic Ca^{2+} mobilisation (Xu*et al.*, 1998). For comparison, the amino acid sequence of the four protease-activated receptors around their cleavage/activation sites given in table 5.

4.1.3 Summary and Aims of this Chapter

4.1.3.1 Summary

The biology of coagulation cascade protease receptors has been reviewed with a particular focus on EPR-1 and PAR-1. This discussion led to the following conclusions:

- In contrast with the growing knowledge of the biology of thrombin and its' receptors, there is limited information available on the cell surface receptors that mediate the cellular effects of fXa. Binding of fXa to EPR-1 is necessary to the stimulation of smooth muscle proliferation*in vitro* and *in vivo*, but it is unknown whether fibroblasts express EPR-1.

- The proteolytic activity of fXa is necessary for the majority of its' cellular effects, including smooth muscle cell proliferation, but the molecular mechanism involved has not been unravelled. FXa can cleave synthetic peptides that mimic the activation site of PAR-1 and PAR-2. However, there is no evidence establishing that fXa activates these receptors.

- There are strong discrepancies between previous reports on the signalling events stimulated by fXa. For instance, the ability of fXa to trigger cytosolic Ca^{2+} mobilisation is unclear. Finally, it is possible that PAR-1 or PAR-2 mediates the cellular effects of fXa.

4.1.3.2 Specific Hypothesis of this Chapter

In this chapter, it was hypothesised that:

> Factor Xa stimulates fibroblast proliferation via binding to EPR-1 and activation of PAR-1.

4.1.3.3 Aims of this Chapter

To address this hypothesis, this chapter has the following aims:

- To assess the mitogenic effect of fXa on fibroblasts in the presence of inhibitors of its' binding to EPR-1.

- To determine whether PAR-1 expression is necessary for the mitogenic effect of fXa on fibroblasts, using genetically engineered mouse fibroblasts deficient for PAR-1.

- To establish whether fXa activates PAR-1 in human fibroblasts by measuring cytosolic Ca^{2+} mobilisation triggered by fXa in resting and in protease-desensitised cells.

4.2 RESULTS

This section aims to characterise the expression of EPR-1 and PAR-1 in human fibroblasts and their role in the mitogenic effects of fXa.

4.2.1 PROTEASE RECEPTOR EXPRESSION IN HUMAN FIBROBLASTS

To begin to identify potential mediators of the mitogenic effects of fXa on human foetal lung fibroblasts, EPR-1 and PAR expression was determined in these cells.

4.2.1.1 Effector-Cell Protease Receptor-1 Expression

Effector-Cell Protease Receptor-1 mRNA Transcripts in Human Foetal Lung Fibroblasts

Figure 4.1 (panel A) shows EPR-1 mRNA transcripts in HFL-1 human foetal lung fibroblasts detected by Northern blot hybridisation. EPR-1 was also detected in these cells by Western blotting followed by enhanced chemiluminescence.

Figure 4.1 (panel B) shows that three proteins immunologically related to EPR-1 were detected with M_r of approximately 52 kDa, 58 kDa and 65 kDa. No band was detected in the absence of primary antibody (data not shown).

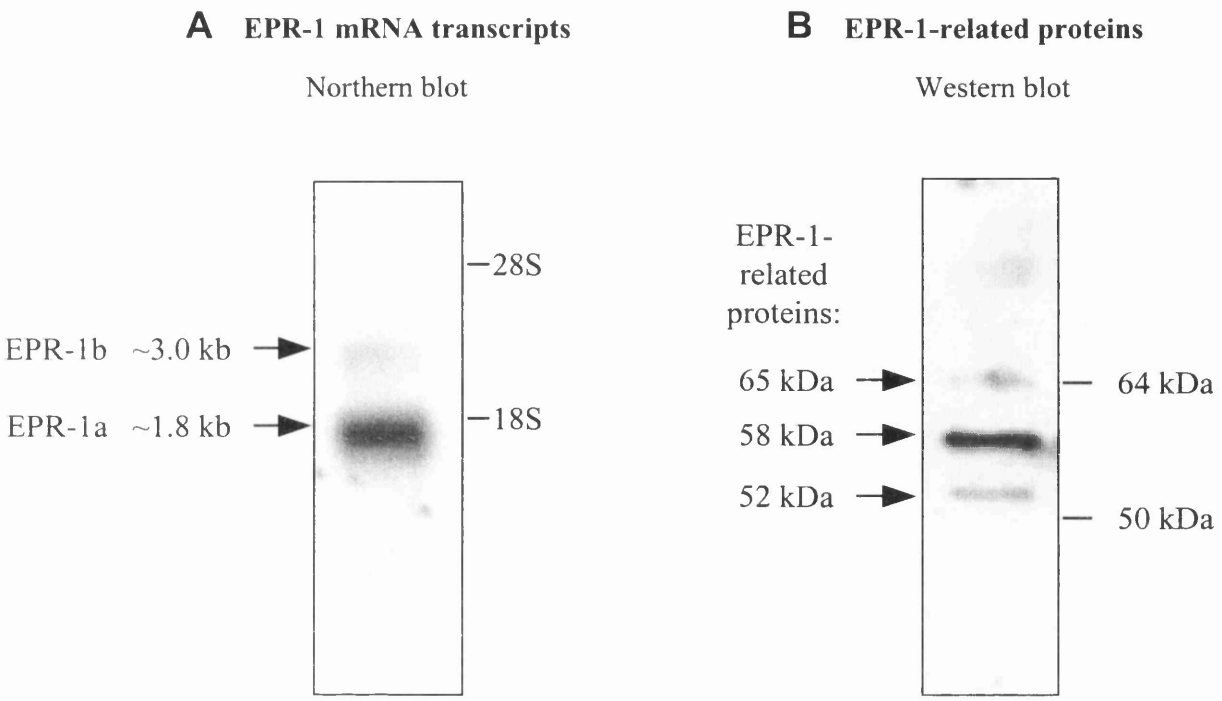


Figure 4.1 **EPR-1 mRNA and protein expression in human foetal lung fibroblasts**

A **EPR-1 mRNA transcripts** were detected by Northern blot hybridisation with a single stand radiolabelled EPR-1 cDNA. Two bands were detected, one of greater intensity and about 1.8 kb, corresponding to the EPR-1a transcript, and one of lower intensity and about 3.0 kb, corresponding to EPR-1b. The positions of the ribosomal RNA transcripts are indicated to the right.

B **EPR-1-related proteins** were detected immunologically on Western blots with mAb B6. Three bands of about 52 kDa, 58 kDa and 65 kDa M_r were detected. The positions of the M_r makers are indicated to the right.

4.2.1.2 Protease-Activated Receptor Expression

The expression of PAR-1, PAR-2, PAR-3 and PAR-4 by human foetal lung fibroblasts was assessed. Human megakaryocytes and platelets express PAR-3 and PAR-4 (Kahn *et al.*, 1998b, Kahn *et al.*, 1999). RNA from the megakaryocytic cell line CHFR-288 was analysed in parallel to the human foetal lung fibroblast RNA as a positive control.

Protease-Activated Receptor-1 Protein in Human Foetal Lung Fibroblasts

Figure 4.2 shows that a single band of about 58 kDa M_r was detected by western blot of human foetal lung fibroblast lysates using an anti-PAR-1 amino terminus.

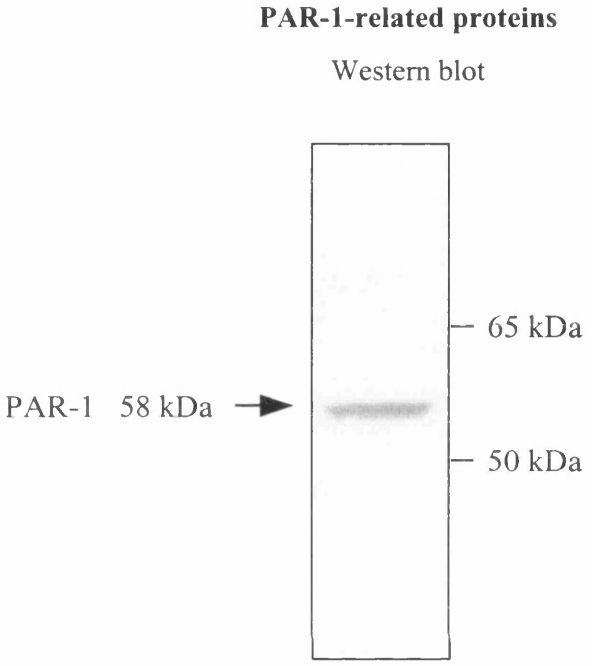


Figure 4.2 PAR-1 in human foetal lung fibroblasts

PAR-1 in human foetal lung fibroblasts was detected immunologically on Western blots with anti-rat PAR-1 mAb. One band was detected of about 58 kDa M_r. The positions of M_r markers are indicated to the right of the figure.

Protease-Activated Receptor mRNA Transcripts in Human Foetal Lung Fibroblasts

Figure 4.3 shows that PAR-1, PAR-2 and PAR-3 mRNA transcripts were detected in human foetal lung fibroblasts with sizes of about 3.5 kb, 3.0 kb and 3.4 kb, respectively. In addition, the PAR-3 transcripts in our fibroblasts and CHFR-288 megakaryocytic cells co-migrated. The 2.1 kb and 6 kb PAR-4 transcripts were totally absent from our fibroblasts. However, a faint band of about 4.7 kb was detected by the PAR-4 cDNA probe.

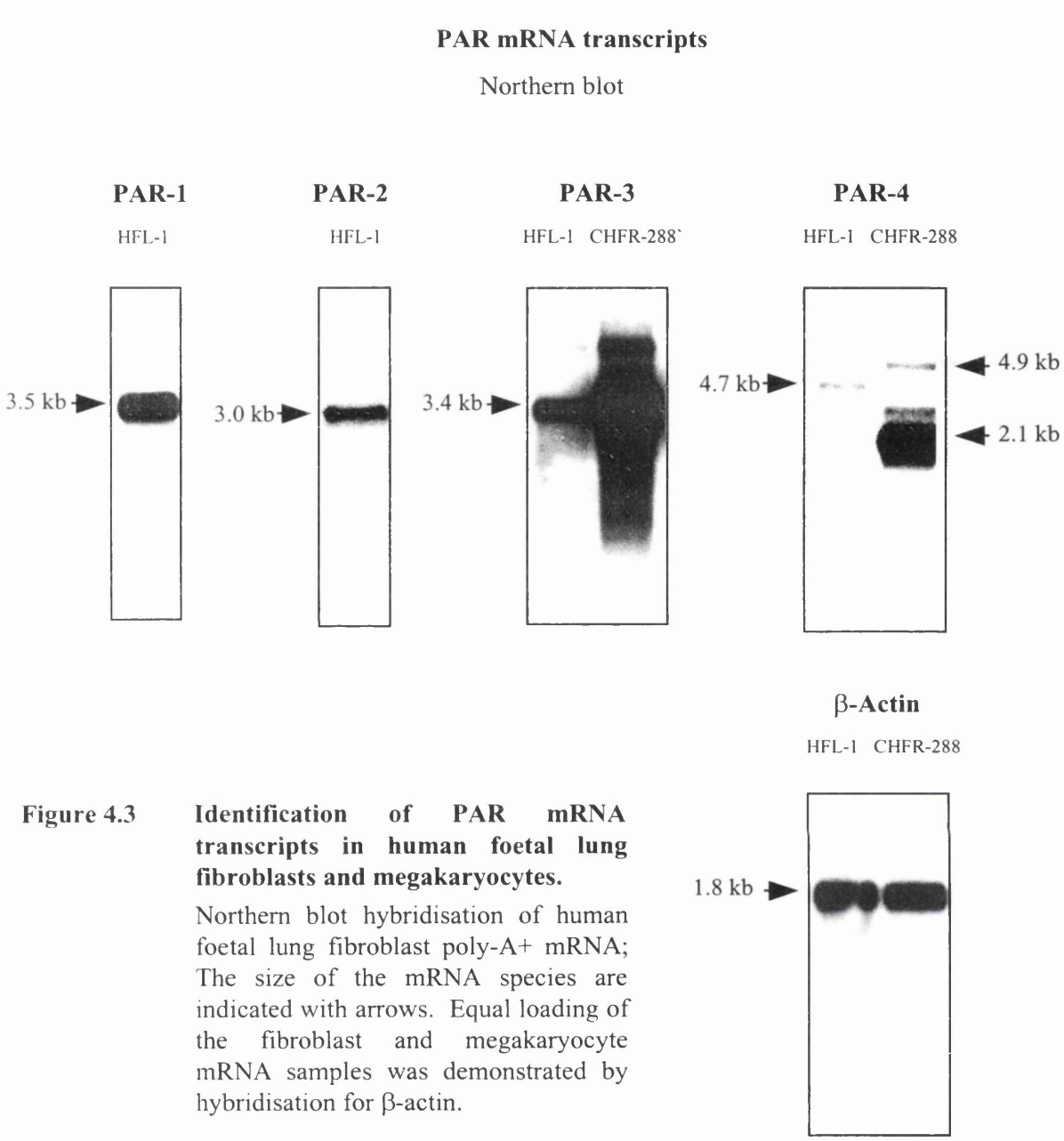


Figure 4.3 **Identification of PAR mRNA transcripts in human foetal lung fibroblasts and megakaryocytes.**

Northern blot hybridisation of human foetal lung fibroblast poly-A+ mRNA; The size of the mRNA species are indicated with arrows. Equal loading of the fibroblast and megakaryocyte mRNA samples was demonstrated by hybridisation for β-actin.

Localisation of Protease-Activated Receptor-1 in Human Foetal Lung Fibroblasts

Figure 4.4 shows the localisation of PAR-1 in human foetal lung fibroblasts that were immunolabelled with the PAR-1 exosite specific mAb WEDE15 and observed by confocal microscopy (panel A). Immunocytological staining was found in resting human fibroblasts and it was located mostly to intracellular vesicles around the nucleus in the region of the endoplasmic reticulum. No significant amounts of fluorescence were detected in fibroblasts treated with non-specific mouse immunoglobulin G that were used as controls (panel B).

A

Fluorescence labelling of human foetal lung fibroblasts with a PAR-1 specific antibody.

B

None immune control shows no staining in human foetal lung fibroblasts.

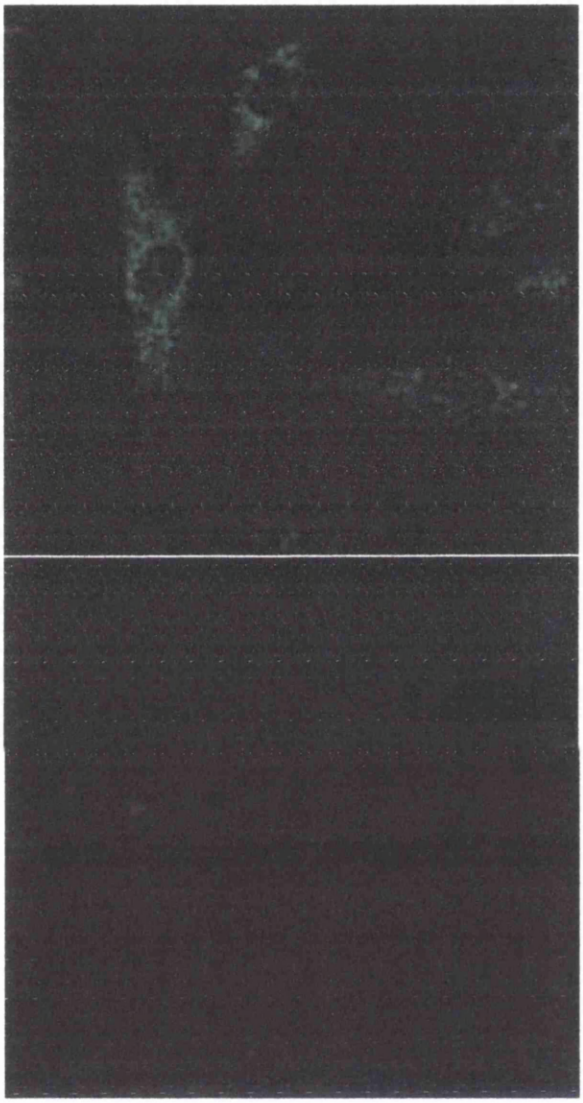


Figure 4.4 PAR-1 localisation in human foetal lung fibroblasts

Resting human foetal lung fibroblasts PAR-1 were labelled immunologically in the presence and absence of a human PAR-1 specific mAb. One μm sections taken across the centre of the cells are presented. In the presence of the PAR-1 specific antibody, the majority of the staining can be observed in intracellular vesicles that may be associated with the fibroblast endoplasmic reticulum.

4.2.2 Effector Cell Protease Receptor-1 Mediates the Mitogenic Effect of Factor Xa in Human Fibroblasts

Figure 4.5 shows the mitogenic effect of fXa on human foetal lung fibroblasts in the presence of anti-fXa (pAb JC15) and anti-EPR-1 neutralising antibodies (mAb B6).

The anti-fXa pAb JC15 inhibited the mitogenic activity of fXa from 120±23% to 39±11% above medium control in the DNA synthesis assay (panel A). The anti-EPR-1 mAb B6 inhibited the mitogenic activity of fXa from 254±13% to 176±3% above medium control in the DNA synthesis assay (panel B).

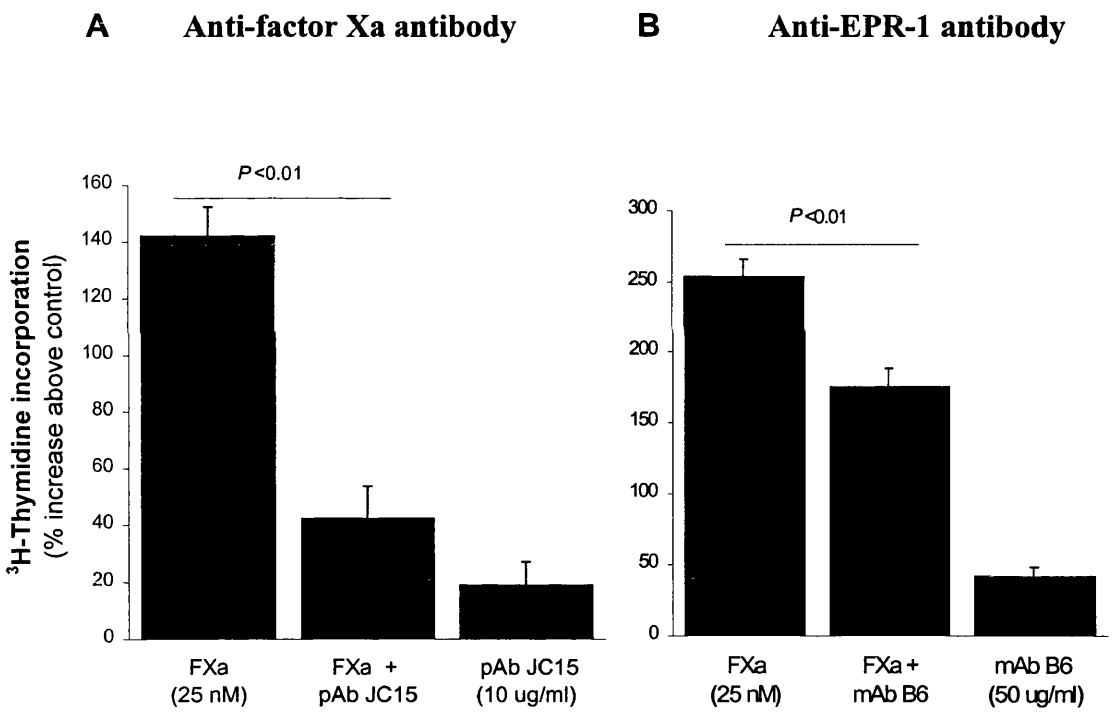


Figure 4.5 **An anti-factor Xa antibody and an anti-EPR-1 antibody inhibit factor Xa-stimulated fibroblast DNA synthesis**

A Effect of pAb JC15 which is directed against residues Leu^{83}-Leu^{88} of fXa on fXa-stimulated fibroblast DNA synthesis.

B Effect of mAb B6 which is directed against residues Pro^{120}-Ala^{154} of EPR-1 on fXa-stimulated fibroblast DNA synthesis. ^{3}H-thymidine uptake assay; expressed in % increase above control ± s.e.m., *p* values are calculated between treatments, representative experiment with 6 replicates for each value.

4.2.3 PROTEASE-ACTIVATED RECEPTOR-1 MEDIATES THE MITOGENIC EFFECT OF FACTOR Xa IN MOUSE FIBROBLASTS

PAR-1-deficient mouse fibroblasts were used to determine whether PAR-1 expression is necessary for fibroblasts to proliferate in response to fXa. The effects of thrombin and mast cell tryptase were measured as positive and negative controls to assess the ability of fibroblasts to proliferate in PAR-1-dependent and PAR-1-independent mechanisms (Molino *et al.*, 1997c).

In wild type control mouse fibroblasts, fXa, thrombin and tryptase elicited mitogenic responses of 95±8%, 86±14% and 61±1% above medium control respectively (all $p<0.05$; figure 4.6). In contrast, PAR-1-deficient mouse fibroblasts failed to respond to fXa and thrombin (7±1% and 7±2% above medium control respectively, $p>0.5$), but retained the capacity to proliferate in response to tryptase (48±3% above medium control, figure 4.6). PAR-1 deficient fibroblasts also retained their full capacity to proliferate in response to 10% NCS (374±76% above medium control, data not shown).

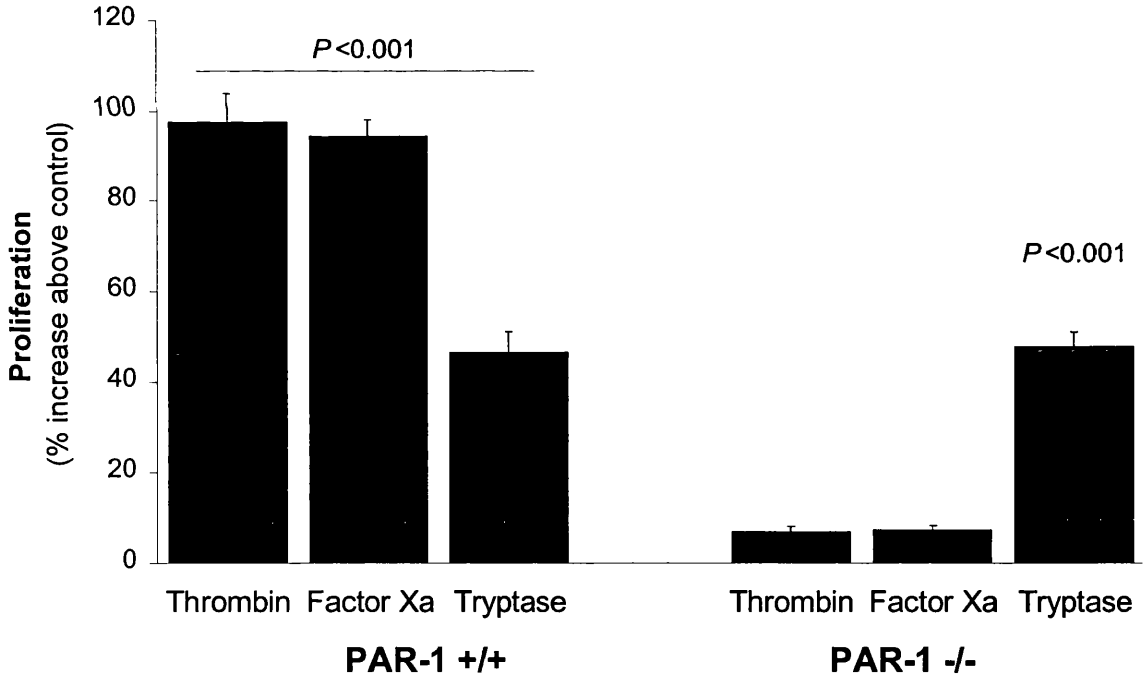


Figure 4.6 **Factor Xa stimulates proliferation in wild type but not PAR-1-deficient mouse fibroblasts**

Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., p values are calculated in comparison with control, mean of 2 experiments with 6 replicates for each value. [factor Xa] = [thrombin] = [tryptase] = 25 nM

4.2.4 PROTEASE-ACTIVATED RECEPTOR-1 MEDIATES CYTOSOLIC CALCIUM MOBILISATION IN RESPONSE TO FACTOR Xa IN HUMAN FIBROBLASTS

In this section, the effect of fXa on cytosolic Ca^{2+} mobilisation by human fibroblasts was determined. Furthermore, it has previously been shown that the Ca^{2+} mobilisation response to PAR-1 agonists can be desensitised. To determine whether PAR-1 mediates the effects of fXa in fibroblasts, the effect of fXa on cytosolic Ca^{2+} mobilisation was determined in cells desensitised with thrombin or fXa.

4.2.4.1 Effect of Factor Xa, Thrombin and Peptide TFLLR on Cytosolic Calcium Mobilization in Human Fibroblasts

The effects of fXa, thrombin and synthetic PAR-1 agonist peptide TFLLR on cytosolic Ca^{2+} mobilisation were measured in human foetal lung fibroblasts preincubated with regular culture medium.

Figure 4.7 (panel A) shows that fXa stimulated a significant increase in intracellular Ca^{2+} mobilisation within 10 to 12 sec of stimulation. The $[Ca^{2+}]_i$ changed from a resting state value of about 70 nM to a maximum of over 225 nM, 60 sec after stimulation. This stimulation corresponds to an increase of about 225% above resting $[Ca^{2+}]_i$ and only one rise and fall in $[Ca^{2+}]_i$, or *Ca^{2+} transient*, was observed over the monitoring period of 240 sec. The average increase in $[Ca^{2+}]_i$ stimulated by fXa was of a lesser maximal amplitude (225 nM versus 360 nM for thrombin) but more sustained than that triggered by thrombin (more than 240 sec versus 200 sec for thrombin; See below).

Panel B shows that thrombin stimulated a significant increase in $[Ca^{2+}]_i$ within 5 sec of stimulation. The $[Ca^{2+}]_i$ changed from a resting state value of about 70 nM to a maximal $[Ca^{2+}]_i$ of over 360 nM, about 60 sec after stimulation. This stimulation corresponds to an increase of about 410% above resting $[Ca^{2+}]_i$ and only one Ca^{2+} transient was observed over the monitoring period of 240 sec.

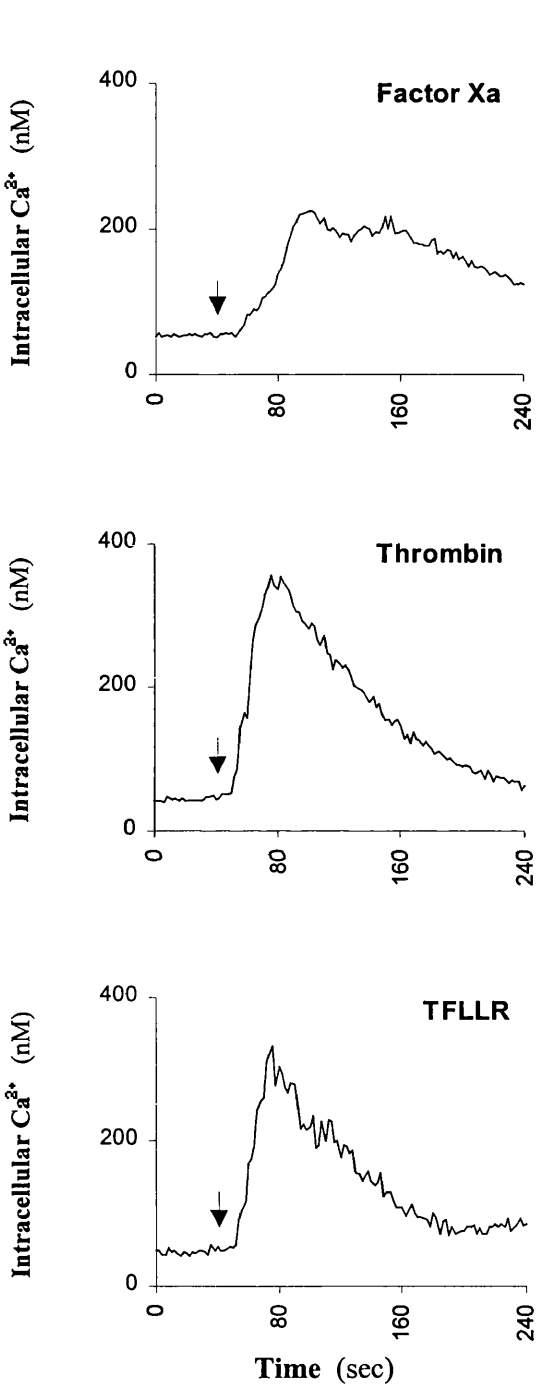


Figure 4.7 **Effect of factor Xa, thrombin and a PAR-1 specific agonist (TFLLR) on $[Ca^{2+}]_i$ mobilization in resting human foetal fibroblasts**

Kinetics of calcium spectrofluorimetry; expressed in nM of Ca^{2+} per cell, mean of 3 independent experiments, each with 10 to 12 cells measured (n=30 to 36). Arrowheads show time of stimulation (40 sec). [fXa] = [thrombin] = 100 nM; [synthetic peptide TFLLR] = 250 μM.

Panel C shows that the PAR-1 specific agonist peptide TFLLR stimulated a significant increase in intracellular Ca^{2+} mobilisation within 5 sec of stimulation. The $[Ca^{2+}]_i$ changed from a resting state value of about 70 nM to a maximal $[Ca^{2+}]_i$ of over 360 nM, about 60 sec after stimulation. This stimulation corresponded to an increase of about 410% above resting $[Ca^{2+}]_i$ and only one rise and fall in $[Ca^{2+}]_i$ was observed over the monitoring period of 240 sec.

4.2.4.2 Proteolytically Inactive Factor Xa does not Stimulate Cytosolic Calcium Mobilization in Human Fibroblasts

To investigate the molecular basis of fXa-stimulated Ca^{2+} mobilisation in human fibroblasts, the effects of catalytic site-inhibited fXa (DEGR-fXa, 100 nM) was investigated on fibroblast Ca^{2+}. Figure 4.8 shows that the basal (resting state) fibroblast $[Ca^{2+}]_i$ was monitored for 40 sec before stimulation. DEGR-fXa does not stimulate intracellular free Ca^{2+} in human fibroblasts over a period of 240 sec, but retain a $[Ca^{2+}]_i$ of about 70 nM over the monitoring period.

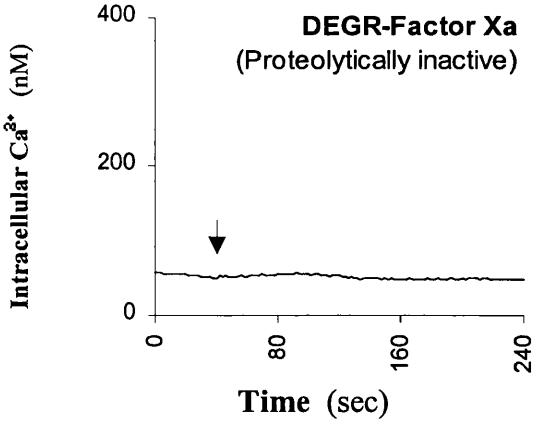


Figure 4.8 **Effect of proteolytically inactive factor Xa (DEGR-factor Xa) on $[Ca^{2+}]_i$ mobilization in resting human foetal fibroblasts**

Kinetics of calcium spectrofluorimetry; expressed in nM of Ca^{2+} per cell, mean of 2 independent experiments, each with 12 cells measured (n=24). Arrowhead shows time of stimulation (40 sec), [DEGR-fXa] = 100 nM.

4.2.4.3 Effect of Factor Xa and Thrombin on Cytosolic Calcium Mobilization in Factor Xa-Desensitised Human Fibroblasts

The effect of fXa on cytosolic Ca^{2+} mobilisation was measured in PAR-1-depleted fibroblasts to determine whether PAR-1 expression is necessary to its'effects. It is known that the incubation of human cells with thrombin for over 60 min is sufficient to completely deplete cell surface PAR-1 and prevent *de novo* PAR-1 expression for another 18 hr (Ellis *et al.*, 1999b). Figure 4.9 shows the effects of fXa and thrombin (100 nM) on cytosolic Ca^{2+} mobilisation after desensitisation with thrombin (25 nM) for 90 min.

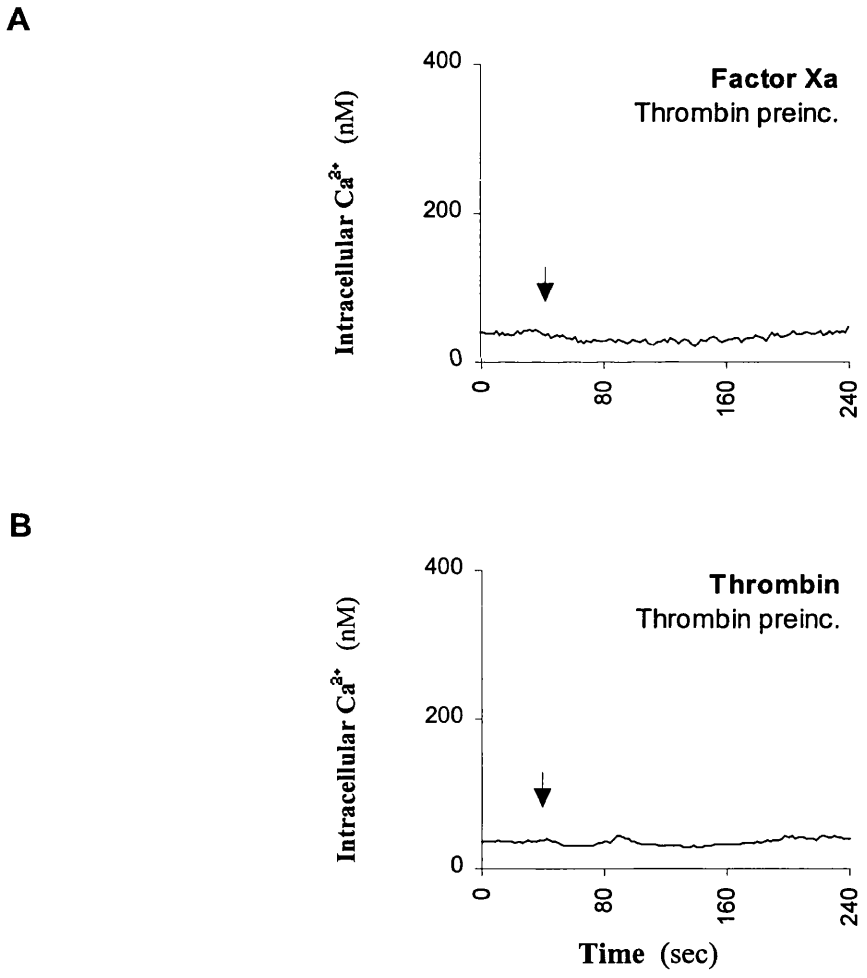


Figure 4.9 **Effect of factor Xa and thrombin on $[Ca^{2+}]_i$ mobilization in thrombin-desensitised human foetal fibroblasts**

Kinetics of calcium spectrofluorimetry; expressed in nM of Ca^{2+} per cell, mean of 3 independent experiments, each with 10 to 12 cells measured (n=30 to 36). Arrowheads show time of stimulation (40 sec). [fXa] = [thrombin] = 100 nM.

Figure 4.9 (panel A) shows that human fibroblasts pre-incubated with thrombin for 90 min do not mobilise Ca^{2+} in response to fXa but retain a $[Ca^{2+}]_i$ of about 70 nM over the monitoring period of 240 sec.

Panel B shows that pre-incubation with thrombin renders fibroblasts unable to mobilise Ca^{2+} in response to thrombin in a phenomenon termed *homologous desensitisation.*

4.2.4.4 Effect of Factor Xa, Thrombin and Peptide TFLLR on Cytosolic Calcium Mobilization in Thrombin-Desensitised Human Fibroblasts

To further investigate the mechanisms of fXa-stimulated Ca^{2+} mobilisation, the effects of fXa (100 nM), thrombin (100 nM) and the PAR-1 selective peptide agonist TFLLR (250 μM) were investigated on fibroblast cytosolic Ca^{2+} mobilisation after desensitisation with fXa (25 nM) for 90 min. The basal (resting state) fibroblast $[Ca^{2+}]_i$ was monitored for 40 sec before stimulation.

Figure 4.10 (panel A) shows that human fibroblasts pre-incubated with fXa for 90 min do not mobilise cytosolic free Ca^{2+} in response to fXa over a period of 240 sec but retain a $[Ca^{2+}]_i$ of about 70 nM over the monitoring period.

Panel B shows that fXa-desensitised fibroblasts retained the capacity to mobilise significantly cytosolic Ca^{2+} in response to thrombin stimulation. The $[Ca^{2+}]_i$ changed within 10 sec of stimulation from a resting value of about 70 nM to a maximal $[Ca^{2+}]_i$ of about 380 nM, 25 to 30 sec after stimulation. This stimulation corresponds to an increase of about 425% above resting state $[Ca^{2+}]_i$ ($p<0.01$), a similar amplitude to that stimulated by thrombin in non-desensitised fibroblasts. Only one Ca^{2+} transient was observed over the monitoring period of 240 sec. Fibroblasts desensitised with fXa restore their resting $[Ca^{2+}]_i$ in less than 120 sec after stimulation. This is twice as fast as the time necessary for non-desensitised fibroblasts.

Panel C shows that human fibroblasts pre-incubated with fXa for 90 min do not mobilise cytosolic free Ca^{2+} significantly in response to the PAR-1 specific agonist peptide TFLLR over a period of 240 sec ($p>0.5$) but retain a $[Ca^{2+}]_i$ of about 70 nM over the monitoring period.

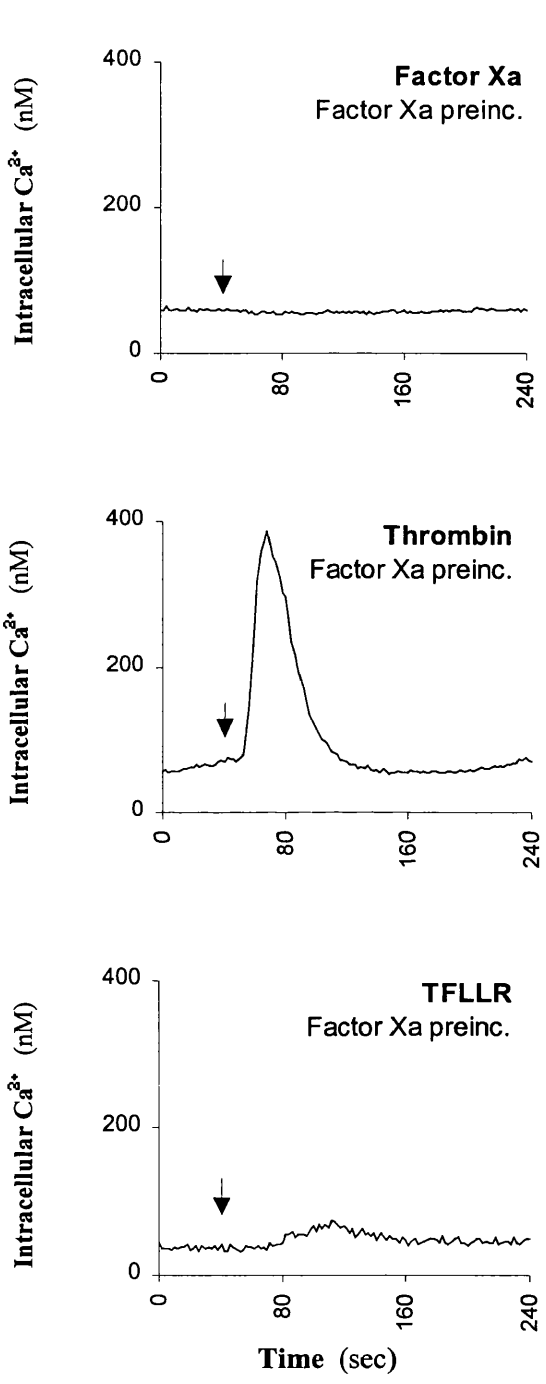


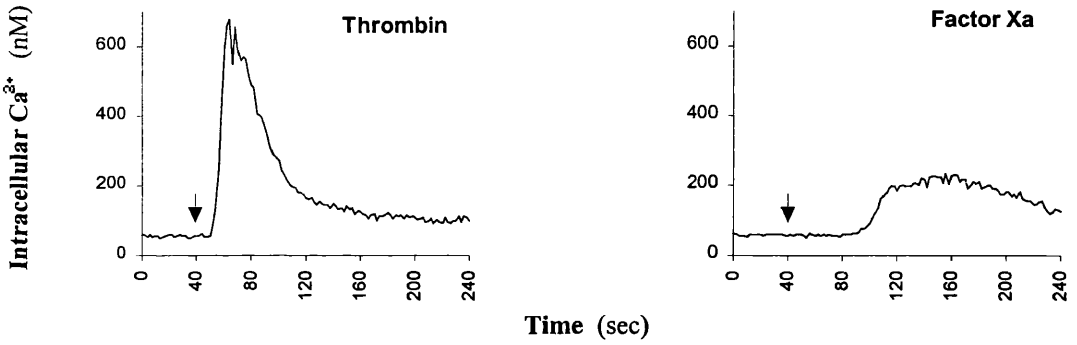
Figure 4.10 **Effect of factor Xa, thrombin and a PAR-1 specific agonist (TFLLR) on $[Ca^{2+}]_i$ mobilization in factor Xa-desensitised human foetal fibroblasts**

Kinetics of calcium spectrofluorimetry; expressed in nM of Ca^{2+} per cell, mean of 3 independent experiments, each with 10 to 12 cells measured (n=30 to 36). Arrowheads show time of stimulation (40 sec). [fXa] = [thrombin] = 100 nM; [synthetic peptide TFLLR] = 250 μM.

4.2.5 PROTEASE-ACTIVATED RECEPTOR-1 MEDIATES CYTOSOLIC CALCIUM MOBILISATION IN RESPONSE TO FACTOR Xa IN MOUSE FIBROBLASTS

PAR-1 deficient mouse fibroblasts were used to determine whether PAR-1 expression is necessary for fibroblasts to mobilise cytosolic free Ca^{2+} in response to fXa. The effect of thrombin was also measured as a positive control.

A Wild type mouse lung fibroblasts



B PAR-1-deficient mouse lung fibroblasts

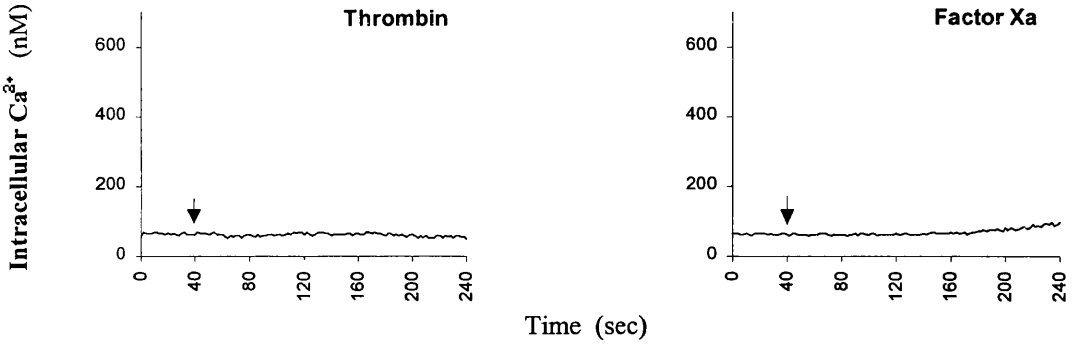


Figure 4.11 Effect of human factor Xa and human thrombin on $[Ca^{2+}]_i$ mobilization in mouse wild type and PAR-1-deficient lung fibroblasts

Kinetics of calcium spectrofluorimetry; expressed in nM of Ca^{2+} per cell, mean of 3 independent experiments, each with 10 to 12 cells measured (n=30 to 36). Arrowheads show time of stimulation (40 sec). [fXa] = [thrombin] = 100 nM.

Figure 4.11 (panel A) shows that fXa elicited a significant increase in cytosolic Ca^{2+} mobilisation within 60 sec of stimulation in wild type control mouse fibroblasts. The $[Ca^{2+}]_i$ changed from a resting state value of about 70 nM to a maximal $[Ca^{2+}]_i$ of about 250 nM, 110 sec after stimulation. This stimulation corresponds to an increase of over 310% above resting $[Ca^{2+}]_i$ and only one rise and fall in $[Ca^{2+}]_i$ was observed over 240 sec. The marked and extended delay in the onset of Ca^{2+} mobilisation is representative of fXa stimulation of all cells within the studied populations. The average increase in $[Ca^{2+}]_i$ stimulated by fXa is of a lesser maximal amplitude (250 nM versus 650 nM for thrombin) but more sustained than that triggered by thrombin in mouse fibroblasts (over 120 sec long versus about 100 sec long for thrombin, see below). In addition, this increase in $[Ca^{2+}]_i$ stimulated by fXa in resting mouse fibroblasts is markedly different from that stimulated in resting human fibroblasts due to the remarkable delay in the onset of the intracellular signal (about 60 sec versus 10 sec in human fibroblasts).

Figure 4.11 (panel A) ahos that thrombin elicited a significant increase in intracellular Ca^{2+} mobilisation within 10 sec of stimulation in mouse wild type fibroblasts. This stimulation corresponds to an increase of over 800% above resting $[Ca^{2+}]_i$ and only one rise and fall in $[Ca^{2+}]_i$ was observed over 240 sec. Although of greater amplitude, this increase in $[Ca^{2+}]_i$ stimulated by thrombin in mouse fibroblasts is not different from that elicited in human fibroblasts in terms of time course.

Figure 4.11 (panel B) shows that no cytosolic Ca^{2+} mobilisation is induced by fXa or thrombin in PAR-1 deficient mouse fibroblasts, The cells retained a $[Ca^{2+}]_i$ of about 70 nM.

4.2.6 Cytosolic Calcium Mobilisation Contributes to the Mitogenic Effect of Factor Xa in Fibroblasts

To determine whether free intracellular calcium mobilisation is linked to the mitogenic effect of fXa in human fibroblasts, the cells were loaded with the cell-permeant cytosolic calcium chelator BAPTA prior to stimulation with fXa (25 nM), see section 2.2.2.4. Fibroblast DNA synthesis was assessed after 20 hr of incubation with the protease (figure 4.12). Human fXa stimulated fibroblast proliferation by about 200% increase above control., whereas BAPTA alone had no significant effect on fibroblast proliferation over the concentration range tested (1 to 100 μM; p>0.01 compared to control). However, BAPTA inhibited fXa-stimulated fibroblast DNA synthesis in a significant and dose-dependent manner between 10 μM and 100 μM.

4.2.7 THE MITOGENIC EFFECT OF FACTOR Xa IN FIBROBLASTS IS MEDIATED BY A G PROTEIN-COUPLED RECEPTOR

To determine whether the mitogenic effect of fXa in human fibroblasts is mediated by a G protein coupled receptor, the cells were loaded with the Gαi inhibitor Pertussis toxin prior to stimulation with fXa (25 nM). After 20 hr of incubation with the protease, fibroblast DNA synthesis was assessed with the ^{3}H-thymidine incorporation assay (figure 4.13).

Human fXa stimulated fibroblast proliferation by about 350% increase above control., whereas Pertussis toxin alone had no significant effect on fibroblast proliferation over the concentration range tested (1 to 100 ng/ml; p>0.01 compared to control). However, Pertussis toxin inhibited fXa-stimulated fibroblast DNA synthesis in a significant and dose-dependent manner between 1 ng/ml and 100 ng/ml.

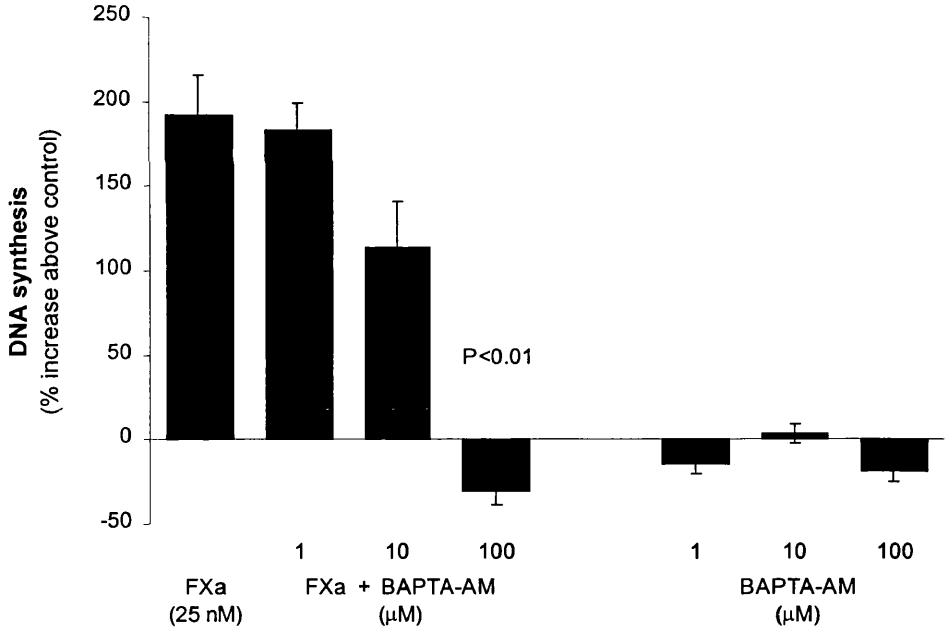


Figure 4.12 **The free cytosolic calcium chelator BAPTA inhibits factor Xa-stimulated fibroblast DNA synthesis**

^{3}H-Thymidine incorporation assay; expressed in % increase above control ± s.e.m., p values are calculated in comparison with stimulation with fXa, representative experiment with 6 replicates for each value.

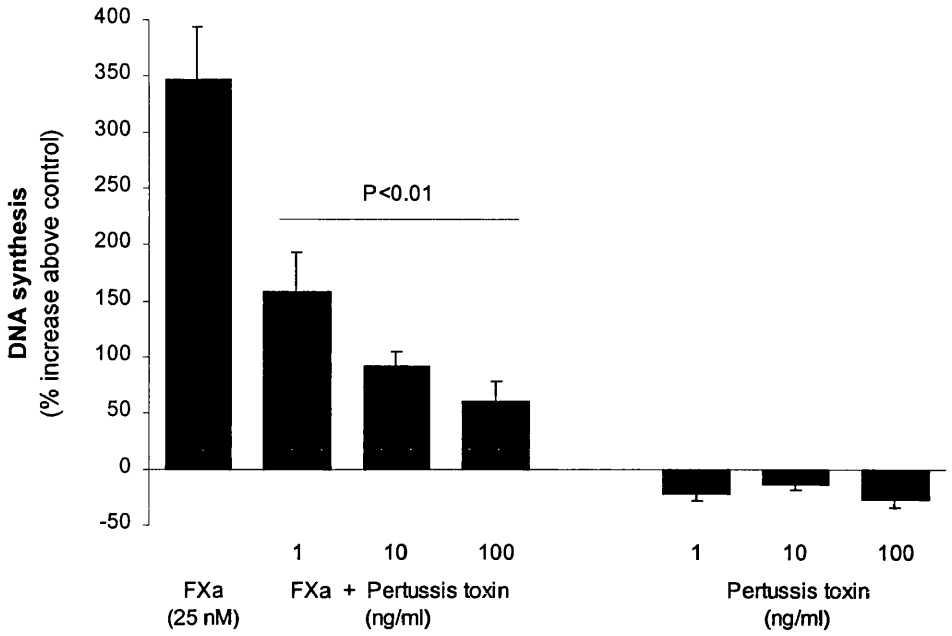


Figure 4.13 **Gαi protein inhibitor Pertussis toxin abrogates factor Xa-stimulated fibroblast DNA synthesis**

^{3}H-Thymidine incorporation assay; expressed in % increase above control ± s.e.m., *p* values are calculated in comparison with stimulation with fXa, representative experiment with 6 replicates for each value.

4.3 DISCUSSION

In chapter 4, It was shown that fXa and thrombin are the two most potent mitogens activated by the extrinsic coagulation pathway and that their effects are identical in terms of kinetics and magnitude. This chapter focused on the cell surface mechanism that mediates the mitogenic effect of fXa.

4.3.1 EFFECTOR-CELL PROTEASE RECEPTOR-1

4.3.1.1 Effector-Cell Protease Receptor-1 Expression by Fibroblasts

Northern blot analysis of human foetal lung fibroblasts revealed two bands of about 1.8 kb and 3.0 kb that corresponded to the sizes of the two mRNA splice variants of EPR-1. Previous studies showed that two forms of mRNA can arise from the EPR-1 gene. In Chinese hamster ovary cells transfected with the EPR-1 cDNA, one transcript (termed EPR-1a) is about 1.5 kb long

and gives rise gives rise to the receptor for fXa, a 337 amino acid protein with a transmembrane domain (Altieri and Edgington, 1990; Altieri, 1994a). In these cells The second splice variant, EPR-1b, is about 1.9 kb (Altieri, 1994b). The existence of the 1.9 kb EPR-1b splice variant was explained by the retention of a 451 bp intronic intervening sequence containing several stop-of-translation signals (Altieri, 1994b) and this transcripts encodes a novel EPR-1-related protein of 110 amino acids only. This truncated protein comprises a nucleus-targeting and it is mostly confined to the nucleus signal (Altieri, 1994b). The function of the EPR-1b protein remains cryptic.

Two EPR-1 mRNA species of 1.8 kb and 3.0 kb have also been reported in activated monocytic cells (Altieri, 1994a), smooth muscle and endothelial cells (Nicholson*et al.*, 1996). In addition, smooth muscle cells also express a 1.3 kb EPR-1 mRNA species (Nicholson *et al.*, 1996). The two EPR-1 transcripts detected in human foetal lung fibroblasts correspond to the two EPR-1 mRNA splice variants previously described.

Immunodetection of EPR-1 by Western blotting of total protein extracts from human foetal lung fibroblasts showed three bands of about 52 kDa, 58 kDa and 65 kDa M_r. They correspond to EPR-1-related proteins described in smooth muscle cells (65 kDa; Nicholson*et al.*, 1996; Herbert *et al.*, 1998), endothelial cells (~54 kDa and 58 kDa; Nicholson *et al.*, 1996), platelets and megakaryocytic cells (~65 kDa; Bouchard *et al.*, 1997), as well as activated monocytic cells (58 kDa and 65 kDa; Altieri and Edgington, 1989). It has been suggested that the different M_r of these EPR-1 proteins may reflect a range of post-translational modifications (Altieri and Edgington, 1989).

Taken together with the finding of EPR-1 mRNA transcripts, these data in human foetal lung fibroblasts support the concept that they express the three forms of EPR-1-related proteins previously reported in other cell types.

4.3.1.2 EPR-1 Mediates the Mitogenic Effect of Factor Xa in Human Fibroblasts

Binding of fXa to its' receptor EPR-1 has previously been described and necessitates residues Leu^{83}-Leu^{88} of fXa to interact with residues Pro^{120}-Ala^{154} of EPR-1 (Ambrosini and Altieri, 1996; Ambrosini *et al.*, 1997). This study shows that a polyclonal antibody directed against the EPR-1-binding region of fXa (pAb JC15) inhibited about 70% of the mitogenic potential of fXa in human foetal lung fibroblasts. Furthermore, a mAb directed against the fXa-binding region of EPR-1 (mAb B6) inhibited up to 30% of this effect. This suggests that binding

of fXa to EPR-1 plays an important role in the effect of the protease on human fibroblast proliferation. Collectively, these data suggest that the proliferative activity of fXa is mediated at least in part, by the interaction between fXa and EPR-1. This is consistent with observations in smooth muscle cells (Herbert *et al.*, 1998) and endothelial cells (Bono *et al.*, 1997). However, the exact nature of the contribution of EPR-1 to the cellular effects of fXa has not been clearly established.

This study also shows that a catalytically inhibited fXa, DEGR-fXa, has little mitogenic effects compared to fXa. It has previously been shown in smooth muscle cells, endothelial cells and other cell types that binding of fXa to EPR-1 is dependent upon activation of the protease, but not on its' catalytic activity (Ambrosini and Altieri, 1996; Bono *et al.*, 1997a). Furthermore, DEGR-fXa retains a conformation similar to that of the active protease, allowing binding to cell surface EPR-1 via residues Leu^{83}-Leu^{88} (Ambrosini and Altieri, 1996). Thus, the lack of effect of DEGR-fXa on fibroblast proliferation suggests that binding of fXa to EPR-1 is not sufficient to stimulate fibroblast proliferation.

Taken together, these results show that binding of fXa to EPR-1 is necessary but not sufficient to promote fibroblast proliferation and that the proteolytic activity of fXa also plays a crucial role in its' cellular effects. In the next section, the expression and the role of protease-activated receptors in the mitogenic effect of fXa on fibroblasts will be discussed.

4.3.2 Protease-Activated Receptors

4.3.2.1 Protease-Activated Receptor Expression by Fibroblasts

PAR-1

A PAR-1 specific probe detected a single transcript of 3.5 kb corresponding to that previously published for PAR-1 in human fibroblasts (Hou *et al.*, 1998) and platelets (Kahn *et al.*, 1999). The detection of PAR-1 mRNA in human foetal lung fibroblasts is also consistent with previous reports that the receptor mediates numerous cellular functions of thrombin in these cells, including proliferation (Connolly *et al.*, 1997) and procollagen production (Chambers *et al.*, 1998; Déry *et al.*, 1998 for review).

Laser-scanning confocal microscopy revealed the presence of PAR-1 in intracellular vesicles that are particularly concentrated around the nuclei of fibroblasts. This is consistent with previous reports in endothelial cells that the majority of PAR-1 is found stored in the plasmalema,

including in the endoplasmic reticulum and golgi apparatus (Hein *et al.,* 1995), and spreading from the perinuclear region to the periphery of the cell (Horvat and Palade, 1995),. Similarly, PAR-1 has been found in intracellular structures that connect with the surface of platelets (Molino *et al.,* 1997a) and this location of the PAR-1 stores is thought to facilitate the rapid expression of the receptor on the cell surface following stimulation. In addition, PAR-1 is thought to be constitutively expressed by fibroblasts and constantly shuttle between intracellular vesicles and the cell surface (Shapiro *et al.,* 1996; Ellis *et al.,* 1999b).

PAR-2

PAR-2 mRNA was detected in human foetal lung fibroblasts and the size of these mRNA species are consistent with those previously reported for PAR-2 (Santulli *et al.* , 1995; Hou *et al.* , 1998). However, PAR-2 has been reported to be absent from gingival (Hou *et al.,* 1998), pulmonary arterial (Belham *et al.,* 1996) or dermal fibroblasts (Santulli *et al.,* 1995). However, it is present in smooth muscle cells, especially in arteries (Molino *et al.,* 1998; Damiano *et al.,* 1999), blood mononuclear cells and neutrophils (Kahn *et al.,* 1999) as judged by Northern and Western blot analysis, reverse transcriptase polymerase-chain-reaction and *in situ* hybridisation. There is additional evidence showing that functional PAR-2 is essentially absent in dermal fibroblasts by measuring Ca^{2+} mobilisation signals in response to PAR-2 agonists (Santulli *et al.,* 1995; Schechter *et al.,* 1998). As a consequence, it may be surprising to identify PAR-2 mRNA in human foetal lung fibroblasts.

However, a recent study showed that proliferating adventitial myofibroblasts express PAR-2 mRNA and that the functional PAR-2 receptor is found on their surface following arterial injury (Damiano *et al.* 1999b). This suggests that PAR-2 may not be constitutively expressed by fibroblasts, but may be upregulated during inflammation and tissue repair. Alternatively, the PAR-2 gene may be constitutively transcribed into mRNA in human fibroblasts, but post-transcriptional events may prevent production of functional PAR-2 protein. Finally, it is also possible that some foetal cells express PAR-2, but not fully differentiated fibroblasts, consistent with a role during development. Nevertheless, my data suggests that PAR-2 mRNA is present in human foetal lung fibroblasts.

PAR-3

This study is the first to report that PAR-3 mRNA is present in human fibroblasts and the PAR-3 mRNA transcript is similar to that present in human megakaryocytic cells. PAR-3 mRNA

was previously identified in splenic megakaryocytic (Kahn *et al.,* 1998b) and pro-monocytic cells (Scase *et al.,* 1997), as well as in the lymph nodes and the bone marrow (Ishihara *et al.,* 1997; Cupit *et al.,* 1999, for review). Recently, it has been suggested that PAR-3 mRNA is consistently found at very low levels in blood mononuclear cells (Kahn *et al.,* 1999) and endothelial cells (Schmidt *et al.,* 1998). In contrast, PAR-3 is not expressed by human platelets and other tissues (Kahn *et al.,* 1999). The relatively weak signal produced fibroblasts RNA compared to megakaryocytic cells suggests that PAR-3 is present at very low levels in human fibroblasts. This is similar to endothelial cells and leukocytes and suggests that PAR-3 is a second thrombin receptor in mesenchymal cells.

PAR-3 mRNA transcripts were identified in mouse platelets, megakaryocytes and in other bone marrow cells (Ishihara *et al.* 1998). Functional PAR-3 is expressed on the surface of mouse platelets and mediates their activation in response to thrombin (Schmidt *et al.,* 1998; Ishihara *et al.,* 1998). This suggests that PAR-3 mediates different functions in human and rodent cells and further studies would be required to determine its' precise function in fibroblasts.

PAR-4

The PAR-4-specific probe detected a faint band of about 4.7 kb in human foetal lung fibroblasts. This transcript clearly differed from the 2.7 kb and 4.9 kb species present in CHFR-288 megakaryocytic cells. Furthermore, the main PAR-4 transcript is about 2.7 kb in most human tissues. However, the three additional bands of about 2.4 kb, 4.2 kb and 6 kb are also found in lung, liver and small intestine (Xu *et al.,* 1998). Thus the PAR-4 mRNA transcript observed in our fibroblasts may correspond to one of those expressed in human adult lungs (Xu *et al.,* 1998),

It is possible that the low hybridisation signal of PAR-4 mRNA in fibroblasts reflects Low expression levels. This would be consistent with previous reports in human platelets (Kahn *et al.,* 1999) and megakaryocytes (Kahn *et al.,* 1998b) where PAR-4 expression amounts to less than 10% of that of PAR-1, PAR-2 and PAR-3. Low PAR-4 mRNA levels have been reported in blood monocytes and lymphocytes and it is absent from neutrophils (Kahn *et al.,* 1999). Thus PAR-4 may not be a predominant receptor in fibroblasts.

Summary

Northern and Western blot analysis as well as confocal microscopy of human foetal lung fibroblasts revealed that they express PAR-1, PAR-2 and PAR-3. A PAR-4 cDNA probe did not detect significant levels of PAR-4 mRNA in human foetal lung fibroblasts.

4.3.2.2 Factor Xa Stimulates Cytosolic Calcium Mobilization in Human Fibroblasts

The effects of fXa, thrombin and the PAR-1 specific agonist TFLLR on cytosolic Ca^{2+} mobilisation were determined by spectrofluorimetry. In resting human foetal fibroblasts, fXa stimulated a significant Ca^{2+} transient within 8 to 12 sec of stimulation. Over 85% of selected cells responded. The $[Ca^{2+}]_i$ changed from a resting value of 70 nM to a maximum of 225 nM about 60 sec after stimulation. This corresponds to an increase in $[Ca^{2+}]_i$ of over 225% above resting levels. Overall, the $[Ca^{2+}]_i$ increase occurred gradually over 60 sec after stimulation. However, it appears as a sharp peak in each individual cell.

This observation correlates with that of Camerer and colleagues in Madin-Darby canine kidney (MDCK) cells, where fXa stimulates a maximal increase in $[Ca^{2+}]_i$ of about 140% above resting levels (Camerer *et al.*,1996). However, these results may not be directly comparable given phenotype differences that exist between kidney cells and fibroblasts. These differences are particularly marked in terms of resting state $[Ca^{2+}]_i$ which average 70 nM in human fibroblasts and 400 nM in MDCK cells (Camerer *et al.*, 1996). In addition, fXa triggered synchronous oscillating signals in MDCK cells over 250 sec, whereas only one transient rise and fall in $[Ca^{2+}]_i$ is observed in human fibroblasts over the same time period. This may suggest distinctive regulation mechanisms for intracellular signalling in response to proteases or differences in protease receptor availability and trafficking between the cell surface and the intracellular stores. Bono and colleagues observed only one rise and fall in $[Ca^{2+}]_i$ stimulated by fXa in human umbilical vein endothelial cells over a period of 300 sec (Bono *et al.*, 1997a). The resting state $[Ca^{2+}]_i$ approximated 70 nM and reached a maximum of up to 185 nM about 60 sec after stimulation (Bono *et al.*, 1997a). These data correspond to that obtained in human foetal lung fibroblasts.

Thrombin stimulated a significant cytosolic Ca^{2+} transient within 5 to 10 sec of stimulation in 100% of the cells that were analysed, consistent with previous observations in human fibroblasts (Molloy *et al.*, 1996). The $[Ca^{2+}]_i$ changed from a basal value of about 70 to 360 nM, about 40 sec after stimulation, suggesting rapid and synchrone activation of the thrombin receptors. The PAR-1 agonist TFLLR provoked an increase in $[Ca^{2+}]_i$ with virtually the same amplitude and kinetics. All tested cells responded similarly to TFLLR. The Ca^{2+} transient stimulated by thrombin corresponds to an increase of over 410% above resting levels in human foetal lung fibroblasts. This correlates with $[Ca^{2+}]_i$ increases stimulated by thrombin in mouse fibroblasts (300% increase, with thrombin at 10 nM; Connolly *et al.*, 1996), in PAR-1-transfected

Xenopus laevis oocytes (420% increase, with thrombin at 20 nM; Vu *et al.,* 1991) or in bovine endothelial cells (600% increase, with thrombin at 100 nM; Garcia *et al.,* 1993).

The average increase in $[Ca^{2+}]_i$ stimulated by fXa is of a lesser maximal amplitude than that triggered by thrombin (225 nM versus 360 nM for thrombin), but sustained for more than 240 sec versus 200 sec for thrombin. This may imply that the mobilisation of cytosolic Ca^{2+} in response to fXa is a more complex mechanism than that of thrombin.

The Proteolytic Activity of Factor Xa Mediates Cytosolic Calcium Mobilisation in Human Fibroblasts

In a previous section, I have shown that fXa stimulates cytosolic Ca^{2+} mobilisation in human fibroblasts. I have also shown that binding of fXa to EPR-1 is necessary for fXa to stimulate fibroblast proliferation. Here, a catalytically inhibited form of fXa (DEGR-fXa) was used as a tool to determine whether binding of fXa to EPR-1 triggers cytosolic Ca^{2+} mobilisation. Results showed that DEGR-fXa has no effect on cytosolic Ca^{2+} mobilisation in fibroblasts in contrast with fXa, suggesting that binding of DEGR-fXa or fXa to EPR-1 is not sufficient to stimulate intracellular signalling in fibroblasts. Furthermore, this implies that the effect of fXa on cytosolic Ca^{2+} mobilisation is dependent on its' intact catalytic activity.

However, EPR-1 is not cleaved by fXa (Altieri and Edgington, 1990; Herbert *et al.*, 1998) and the specific role of the catalytic site of fXa remains unclear.

4.3.2.3 PAR-1 Mediates Cytosolic Calcium Mobilization in Response to Factor Xa in Human Fibroblasts

In the introduction to this chapter, the intracellular signalling events linked to PAR activation, including cytosolic Ca^{2+} mobilisation, were reviewed. Here, the mobilisation of cytosolic Ca^{2+} in response to specific PAR-1 agonists will be used as a marker for the activation of the receptor in human foetal lung fibroblasts. The role of PAR-1 in the mechanism of action of fXa on the fibroblast surface will be dissected in cells desensitised by prolonged exposure to thrombin. Note that in all experiments, agonists were used at a concentration at least four times higher than that of the desensitising protease, thereby increasing the probability of observing a Ca^{2+} signal should any intact PAR-1 remain on the cell surface.

Thrombin-Desensitised Human Foetal Fibroblasts

The effects of fXa, thrombin and a PAR-1 specific agonist (TFLLR) on cytosolic Ca^{2+} mobilisation in thrombin-desensitised human foetal fibroblasts were determined. After 90 min desensitisation with thrombin, the resting $[Ca^{2+}]_i$ was comparable to that of non-desensitised fibroblasts (about 70 nM), suggesting that the fibroblasts were not stimulated by thrombin any more and completely desensitised.

In thrombin-desensitised fibroblasts, thrombin did not stimulate cytosolic Ca^{2+} mobilisation. This confirms that fibroblasts are subject to homologous desensitisation by thrombin, consistent with previous reports in human fibroblasts (Kahn *et al.*, 1999), human kidney cells (Kawabata *et al.*, 1999), megakaryocytic cells (Hoxie *et al.*, 1993) and MDCK cells (Camerer *et al.*, 1996). This is a characteristic feature of proteases that stimulate cytosolic Ca^{2+} mobilisation via protease-activated receptors (section 4.1). It has also been reported previously that thrombin-desensitised cells do not respond to the specific PAR-1 agonists such as TFLLR (Santulli *et al.*, 1995).

Similarly to thrombin, fXa did not stimulate cytosolic Ca^{2+} mobilisation in thrombin-desensitised fibroblasts. This suggests that fXa triggers cytosolic Ca^{2+} mobilisation and intracellular signalling via a cell surface receptor that is desensitised by thrombin in a fashion characteristic of protease-activated receptors. This observation rules out the possibility that the effect of fXa results from PAR-2 activation since PAR-2 is not simulated by thrombin.

At least two thrombin receptors were identified in human foetal lung fibroblasts, PAR-1 and PAR-3 (section 4.3.1.2) and they are both potential candidates for a role in the signalling pathway of fXa. The effects of TFLLR on cytosolic Ca^{2+} mobilisation in fXa-desensitised fibroblasts were.

Factor Xa-Desensitised Human Fibroblasts

The effects of fXa, thrombin and TFLLR on cytosolic Ca^{2+} mobilisation in fXa-desensitised human foetal fibroblasts were evaluated in order to determine if PAR-1 is activated by fXa. After 90 min incubation with fXa, the resting $[Ca^{2+}]_i$ was comparable to that of non-desensitised fibroblasts (about 70 nM), suggesting that the fibroblasts were not being stimulated by fXa any more and totally desensitised.

In fXa-desensitised fibroblasts, fXa did not stimulate cytosolic Ca^{2+} mobilisation, suggesting that fibroblasts are subject to homologous desensitisation by fXa. This novel observation is characteristic of proteases stimulating protease-activated receptors and differs from

the results of Camerer and colleagues (Camerer *et al.*, 1996) in Madin-Darby canine kidney cells (MDCK) who observed little or no homologous desensitisation in response to fXa. However, total desensitisation is only achieved after a sufficiently long incubation of the cells in the presence of a protease such as thrombin (Ellis *et al.*, 1999). It has been reported that more than one hr of incubation with thrombin (25 nM) is necessary to obtain full desensitisation in human endothelial cells (Ellis *et al.*, 1999). Camerer and colleagues desensitised MDCK cells with fXa for one hr before stimulation and it is possible that such a time is not sufficient in these cells which may have different characteristics from endothelial cells. In this study, it was confirmed that one hr of incubation with fXa is not sufficient to desensitise fibroblasts (data not shown), hence a 90 min incubation period was used to produce complete desensitisation of the fibroblasts.

PAR-1-specific synthetic peptides such as TFLLR are known to activate both cleaved and uncleaved PAR-1 on the surface of cells (Hammes and Coughlin, 1999). TFLLR triggers a sharp cytosolic Ca^{2+} mobilisation similar to thrombin in resting fibroblasts, but it did not stimulate cytosolic Ca^{2+} mobilisation in fXa-desensitised fibroblasts. This suggests that no significant levels of PAR-1 are present on the surface of fXa-desensitised fibroblasts and it is consistent with the total desensitisation of PAR-1 by fXa. This also supports the idea that fXa proteolytically activates PAR-1 to produce a state of desensitisation.

In contrast with fXa, thrombin did stimulate a very short cytosolic free Ca^{2+} transient in fXa-desensitised fibroblasts. This thrombin-induced transient had a similar amplitude in fXa-desensitised and resting fibroblasts (maximal increase of about 360 nM). However, the kinetics of thrombin-induced cytosolic free Ca^{2+} transients were markedly altered, suggesting that pre-incubation with fXa changes the ability of human fibroblasts to respond to thrombin. The cytosolic free Ca^{2+} transient was brief and reached its' maximum about 15 sec after stimulation. The $[Ca^{2+}]_i$ was back to baseline within 65 sec after stimulation versus more than 200 sec in resting fibroblasts, or over three times longer. This capacity of fXa-desensitised fibroblasts to mobilise cytosolic free Ca^{2+} in response to thrombin may be interpreted in several manners. This may be the result of incomplete desensitisation, or reflect the ability of thrombin to stimulate cytosolic Ca^{2+} mobilisation via more than one receptor. This will be discussed in detail below.

Effects of Thrombin in Factor Xa-Desensitised Human Fibroblasts

Hein and colleagues reported that the repetition of thrombin challenges (10 nM) of endothelial cells at one hr intervals shortens the time span of their corresponding Ca^{2+} transients. At the first challenge, the transient lasts 80 sec, then 60 sec only at the second challenge and down

to 40 sec at the third challenge (Hein *et al.*, 1994). The amplitude of these transients remained the same at all times (about 500% increase in $[Ca^{2+}]_i$). The thrombin Ca^{2+} transient was similarly shortened after incubation with fXa. This may suggest that PAR-1 receptors were indeed activated by fXa but that they were desensitised slower after exposure to fXa rather than thrombin. There may be other reasons why fXa did not totally desensitise fibroblasts to thrombin. Different conditions may be required for fXa versus thrombin to deplete cell surface PAR-1 in our experimental conditions in our cells. However, the time of incubation (90 min) and the concentration of fXa (25 nM) were sufficient to completely desensitise fibroblasts for the selective PAR-1 agonist peptide TFLLR. This confirms that the experimental conditions employed produced complete PAR-1 desensitisation in human foetal lung fibroblasts.

Alternatively, these data may reflect the existence of several populations of receptors through which thrombin stimulates cytosolic Ca^{2+} mobilisation in our fibroblasts. Some of these receptors may be desensitised by fXa and experimental results obtained with TFLLR help identify one of these receptors as PAR-1. However, there may be other thrombin receptors that are not activated or desensitised by fXa. These receptors may include PAR-3 or PAR-4 or other receptors. Indeed, it has previously been reported that fXa is unable to activate PAR-3 *in vitro* (Ishihara *et al.*, 1997) whereas it activates PAR-1 in PAR-1-expressing *Xenopus laevis* oocytes. In these cells, fXa activates PAR-1 with an efficiency level of about 35% of that of thrombin and probably, in the absence of EPR-1 (Ishihara *et al.*, 1997). Thus, it is possible that fXa activates and desensitises PAR-1 but not PAR-3. Finally, section 4.3.2.1 showed that human foetal lung fibroblasts express PAR-3 and the remaining Ca^{2+}-mobilising potential of thrombin in fXa-desensitised fibroblasts may result from PAR-3 activation.

There is also a possibility that thrombin stimulates on cytosolic Ca^{2+} mobilisation in fXa-desensitised fibroblasts via an unknown receptor. Thrombin has been reported to upregulate tyrosine kinase activation and annexin V mRNA levels in fibroblasts (Sower *et al.*, 1999), endothelial cell proliferation (Herbert *et al.*, 1994) and stimulate wound healing (Carney *et al.*, 1992) via a receptor independent from protease-activated receptors and termed *non-PAR*. This non-PAR putative receptor appears to be activated by peptides mimicking thrombin residues 508 to 530, a domain of thrombin that is devoid of proteolytic activity (Glenn *et al.*, 1988; Carney *et al.*, 1992; Herbert *et al.*, 1994; Sower *et al.*, 1999). These effects of thrombin can not be recapitulated by specific PAR-1 agonists.

Summary

The experimental data gathered in this thesis demonstrate that PAR-1 plays a central role in the signal transduction mechanism of fXa in fibroblasts although all the receptors that mediate cytosolic Ca^{2+} mobilisation in response to thrombin have not been identified.

4.3.2.4 PAR-1 Mediates the Mitogenic Effect of Factor Xa in Mouse Fibroblasts

In the previous section, PAR-1 has been implicated in the signalling receptor for fXa on the surface of human fibroblasts. To confirm the hypothesis that PAR-1 plays a critical role in the cell surface receptor system for fXa, the mitogenic effect of fXa has been studied in wild type and PAR-1-deficient mouse lung fibroblasts.

Thrombin, fXa and mast cell tryptase all stimulated wild type mouse lung fibroblast proliferation. Consistent with previous reports (Vu *et al.,* 1991), mouse foetal lung fibroblasts lacking PAR-1 did not proliferate in response to thrombin. However, PAR-1-deficient mouse lung fibroblasts proliferated in response to mast cell tryptase. This protease is a PAR-2 agonist in human fibroblasts (Mirza *et al.*, 1997) and stimulates fibroblast proliferation and procollagen gene expression (Abe *et al.*, 1998). In contrast, PAR-1-deficient mouse fibroblasts do not proliferate in response to fXa, similarly to thrombin. These data suggest that PAR-1 expression is necessary for fibroblasts to proliferate upon stimulation with fXa. This provides the first experimental evidence that fXa stimulates fibroblast proliferation in a PAR-1-mediated and PAR-2-independent mechanism. These results and the data on EPR-1 (sections 4.3.1.2 and 4.3.1.3) imply that two cell surface receptors are necessary for the mitogenic effect of fXa; EPR-1 and PAR-1.

However, discrepancies exist between humans and rodents regarding the type of protease-activated receptor involved in physiological events such as platelet activation. For instance, PAR-3 mRNA transcripts were identified in mouse platelets, megakaryocytes and bone marrow (Schmidt *et al.,* 1998). Functional PAR-3 is expressed on the surface of mouse platelets (Ishihara *et al.* , 1998) and it seems to play a predominant role in rodent platelet activation in response to thrombin (Kahn *et al.,* 1998b). In contrast, PAR-3 mRNA is mostly absent from human platelets (Ishihara *et al.,* 1997) and PAR-1 and PAR-4 mediate platelet activation in response to thrombin in humans (Kahn *et al.,* 1999). These observations prompt questions regarding the interpretation of experimental results obtained with mouse cells. However, the present findings in mouse cells are consistent with those obtained in human cells and demonstrate that PAR-1 is a critical mediator of the effects of fXa in fibroblasts.

4.3.2.5 PAR-1 Mediates Cytosolic Calcium Mobilization in Response to Factor Xa in Mouse Fibroblasts

The previous sections shows that the mechanism of action of fXa may involve binding to EPR-1 and activation of PAR-1 in human fibroblasts. This section aims to confirm this finding in wild type and PAR-1-deficient mouse fibroblasts.

Wild Type Mouse Lung Fibroblasts

In mouse fibroblasts, thrombin stimulated immediate transient cytosolic Ca^{2+} mobilisation within five sec of stimulation in 100% of the cells selected. The increase in $[Ca^{2+}]_i$ of over 750% above resting levels within 20 sec of stimulation correlates with previous observations in wild type mouse fibroblasts (Connolly *et al.*, 1996). Human fXa stimulated cytosolic Ca^{2+} mobilisation in about 85% of the cells selected. Compared to thrombin, this increase in cytosolic Ca^{2+} mobilisation was delayed by about 55 sec and of a lesser amplitude, about 220% above resting levels. The marked delay in the stimulation cytosolic Ca^{2+} mobilisation by fXa versus thrombin may reflect a greater complexity in the mechanism of its' effect. This lag may reflect the binding of fXa to a first receptor, such as EPR-1, before PAR-1 activation occurs. It is also possible that the species difference between human fXa and the mouse receptors may hamper the normal interaction between these molecules and delay signalling. A rodent form of EPR-1 has not been identified to date. However, peptide mimics of EPR-1 have been successfully used in rats to inhibit the effects of fXa, suggesting that a functional rodent equivalent of EPR-1 exists and recognises human fXa (Cirino *et al.*, 1997).

PAR-1-Deficient Mouse Lung Fibroblasts

Thrombin did not stimulate cytosolic Ca^{2+} mobilisation in PAR-1-deficient mouse fibroblasts, in agreement with previous reports (Connolly *et al.*, 1996). Similarly, fXa did not stimulate cytosolic Ca^{2+} mobilisation in these cells. This supports the idea that PAR-1 plays a role in the cellular effects of fXa in mouse fibroblasts, as suggested in human cells.

Since PAR-1-deficient fibroblasts are totally unable to mobilise cytosolic Ca^{2+} in response to thrombin or fXa, these data also imply that PAR-1 is the only thrombin receptor to mediate these effects in mouse wild type fibroblasts. Thus, data in human and mouse fibroblasts converge to show that the effect of fXa is mediated by PAR-1 activation. However, functional

studies of cytosolic Ca^{2+} mobilisation support the idea that human fibroblasts may express more than one thrombin receptor, including PAR-3 (section 4.3.2.2).

4.3.3 The Mitogenic Effect of Factor Xa in Fibroblasts is Mediated by a Gαi Protein-Coupled Receptor and Cytosolic Calcium Mobilisation

A cytosolic free Ca^{2+} chelator and a Gαi protein inhibitor were employed to further characterise the signalling events triggered by fXa leading to fibroblast proliferation.

The depletion of cytosolic free Ca^{2+} with BAPTA in fibroblasts completely inhibited the mitogenic effect of fXa in a dose response manner. BAPTA was used at a concentration of 1 μM upwards and total inhibition of fibroblast proliferation in response to thrombin was obtained at 100 μM. A rapid rise in cytosolic free Ca^{2+} is a common signalling event in fibroblasts responding to the mitogenic stimuli of growth factors (Wahl and Gruenstein, 1993). Indeed, cytosolic free Ca^{2+} is a permissive factor that commands the entry of fibroblasts into S phase and a key element of the main signalling pathways stimulated by thrombin in fibroblasts (section 4.1.2.2 and Exton, 1997, for review). Cytosolic Ca^{2+} signalling can be abrogated with BAPTA (Wahl and Gruenstein, 1993; Back *et al.*, 1993) and BAPTA inhibits both PDGF- and serum-induced fibroblast DNA synthesis (Wahl and Gruenstein, 1993; Back *et al.*, 1993). This thesis suggests that cytosolic Ca^{2+} signalling plays a role in the mitogenic effects of fXa on fibroblasts, similarly to thrombin.

It is possible to speculate that cytosolic Ca^{2+} mobilisation might promote fXa signalling by stimulating a number of events also linked to PAR-1 activation by thrombin. These include the activation and translocation to the membrane of Ca^{2+}-dependent isoforms of protein kinase C (Ha and Exton, 1993; Heemskerk *et al.*, 1994), phospholipase D (Garcia *et al.*, 1991; Garcia, 1992) and MAPK (Trejo *et al.*, 1996; Meloche *et al.*, 1997).

Pertussis toxin inhibited about 85% of the mitogenic effect of fXa in human fibroblasts. This implies that a heterotrimeric G protein of the Gαi subfamily regulates the stimulation of human foetal lung fibroblast proliferation by fXa. Furthermore, the mitogenic effect of thrombin on fibroblasts is also susceptible to Pertussis toxin (van Corven *et al.*, 1993; Chen *et al.*, 1994; Trejo *et al.*, 1996; Meloche *et al.*, 1997). Some groups even report that treatment with Pertussis toxin inhibits up to 95% of this effect (Meloche *et al.*, 1992). Finally, EPR-1 has not been shown to be a G protein-coupled receptor (Altieri, 1994a). Thus, these data are consistent with the involvement of a second receptor in the mitogenic effect of fXa in fibroblasts suggest that a similar type of receptor mediates the effects of thrombin and fXa.

Summary

Pertussis toxin and BAPTA inhibited the majority of the mitogenic effect of fXa on fibroblasts, implying that Gαi proteins and cytosolic Ca^{2+} mobilisation are central to the signal transduction of fXa. These observations are similar to those obtained with thrombin and entirely consistent with the idea that fXa activates a PAR-1 in human fibroblasts.

4.3.4 EPR-1 AND PAR-1: A NOVEL DUAL RECEPTOR SYSTEM FOR FACTOR Xa

This thesis has shown that binding of fXa to EPR-1 is necessary but not sufficient to stimulate fibroblast proliferation (section 3.2.3.3) and intracellular signalling (section 4.2.3.4). This implied that an additional cell surface receptor may be involved in the effects of fXa, consistent with previous observations in smooth muscle (Herbert *et al.*, 1998) and endothelial cells (Nicholson *et al.*, 1996; Bono *et al.*, 1997a).

The data presented strongly suggests that the second receptor that mediates the mitogenic effects of fXa is PAR-1. The hypothesis that PAR-1 contributes to the mitogenic effects of fXa had previously been excluded on the basis that the thrombin-specific inhibitor hirudin does not affect the effects of fXa in endothelial cells (Nicholson *et al.*, 1996). Indeed, thrombin interacts with a region of PAR-1 that mimics hirudin and this inhibitor is thought to impair the interaction between thrombin and PAR-1 in a competitive manner (Hayes and Tracy, 1999). It is possible that fXa interacts with PAR-1 via a different mechanism that does not involve the hirudin-like region of PAR-1. Furthermore, EPR-1 may contribute to the localisation of fXa on the cell surface, as it does in several cell types (section 4.1.1). Further experiments would be needed to characterise fully the protein-protein interactions involved.

A major difference between thrombin and fXa is that thrombin is thought to cleave PAR-1 without the contribution of cofactors or additional receptors. In contrast, fXa appears to require binding to EPR-1 to activate PAR-1 (section 4.2.2). Binding of fXa to EPR-1 may help bring fXa in close proximity with its' potential substrates on the cell surface such as PAR-1 and enhance its' activation. Binding to EPR-1 is known to confer fXa with prothrombinase activity in the absence of factor Va, suggesting that EPR-1 may play the role of a cofactor (Bouchard *et al.*, 1997). This is supported by the fact that EPR-1 and factor Va share immunological similarities that formed the basis of the discovery of EPR-1 (Altieri and Edgington, 1989; Altieri and Edgington, 1990). I speculate that EPR-1 might function as a cofactor for fXa that promotes PAR-1 cleavage.

Examples of cell surface receptors that alter the affinity of proteases for certain substrates exist. Thrombomodulin is a cell, surface receptor for thrombin with anticoagulant properties (Shirayoshi *et al.,* 1993). It regulates the molecular recognition of protein C by thrombin and promotes the generation of active protein C (DeCristofaro, 1996). Simultaneously, thrombomodulin downregulates the mitogenic effect of thrombin, presumably by changing the affinity of thrombin from FGN and PAR-1 to protein C (Lafay *et al.*, 1998). It is possible that EPR-1 plays a similar role by promoting the recognition and activation of PAR-1 by fXa. In such a model, EPR-1 would promote the mitogenic effects of fXa in cells that also express PAR-1.

Summary

This section has shown that the mitogenic effects of the fXa on fibroblasts are mediated by two main events on the cell surface: Binding to EPR-1 and activation of PAR-1. Furthermore, the stimulation of fibroblast with fXa leads to Gαi protein signalling and cytosolic Ca^{2+} mobilisation. I speculate that EPR-1 may have a similar function in the stimulation of fibroblast proliferation as it does during blood coagulation by promoting the assembly of the prothrombinase complex and the proteolytic activity of fXa on the cell surface. I further suggest that PAR-1 activation is key to intracellular signalling in response to fXa in fibroblasts.

Finally, the finding that fXa stimulates fibroblast proliferation via a novel dual receptor system involving EPR-1 and PAR-1 has implications on our understanding of serine protease receptors and signalling. These issues will be discussed in the broader setting of the regulation of fibroblast function by proteases in chapter 6.

CHAPTER 5

THE EFFECTS OF FIBRINOGEN CLEAVAGE PRODUCTS ON FIBROBLASTS

The proteolysis of FGN during blood coagulation and fibrinolysis results in the formation of a range of soluble cleavage products. Little is known about their mitogenic potential. In this chapter, it is hypothesised that cleavage products of FGN generated at sites of tissue injury stimulate fibroblast proliferation and ECM production *in vitro*. FGN cleavage products will be prepared by extraction from FBN clots or cation exchange chromatography and their effects will be assessed on fibroblast proliferation and protein synthesis.

5.1 INTRODUCTION

FGN cleavage products generated during tissue repair and their cellular effects will be reviewed below. This includes mainly FGN, FBN, FGN cleavage products generated by thrombin and FBN degradation peptides produced by plasmin.

5.1.1 FIBRINOGEN-DERIVED PEPTIDES

5.1.1.1 Fibrin Deposition and Fibrinogen Cleavage Products

Blood coagulation at sites of tissue injury involves the specific processing of blood-borne FGN by thrombin (Stubbs *et al.*, 1992; chapter 1). It transforms soluble FGN into FBN monomers that polymerase spontaneously into an insoluble FBN scaffold (Mosseson, 1998 for review; section 1.2.3 and diagram 5, page 47).

Thrombin cleaves successively fibrinopeptides A and B from the amino termini of Aα– and Bβ–chains (; Bilezikian *et al.*, 1975; Lewis *et al.*, 1985). The FBN monomers polymerise spontaneously into fibrils following an assembly process of intermolecular D to E associations (ends-to-middle). These linear and branched double-stranded FBN fibrils were termed *protofibrils*

(Mosesson, 1997) and their study by electron microscopy suggests that their structure is twisted and not precisely ordered (Medved' *et al.,* 1990). The order of successive fibrinopeptide cleavage (fibrinopeptides A first and then B) and the twist in the structure of the early protofibrils are all thought to promote lateral assembly of the early protofibrils into thicker fibre bundles (Weisel*et al.,* 1987a; Weisel *et al.,* 1987b). The carboxy termini of the α-chains are also thought to contribute to the erection of the FBN scaffold (Wiesel and Papsun, 1987; Gorkun *et al.,* 1994).

FGN also contains non-substrate binding sites for thrombin (Mosesson *et al.,* 1989). Residues β15-42 of the FBN β-chain play a key role in the binding of thrombin and it is thought that the sequestration of thrombin within the FBN clot helps limit the diffusion of the protease outside of the area of trauma (Siebenlist *et al.,* 1990).

Subsequently, factor XIIIa cross-links the FBN fibres which results in increased fibre size and density and promotes the elastic resistance of the FBN scaffold (Carr *et al.,* 1987). Finally, the fibre bundles are also more resistant to protease digestion (Weisel, 1986).

Finally, recent studies have shown that a wide range of FGN cleavage products is generated by the action of thrombin on FGN *in vitro* (Gray *et al.,* 1990). Indeed, peptides that differ from FBN monomers or fibrinopeptides A and B can be observed in the supernatant of FBN clots formed *in vitro*. They have with a great variety of M_r but little is known about their mode of formation or their sequence (Gray *et al.,* 1990; Gray *et al.,* 1995a).

Summary

Thrombin is classically thought to generate several categories of FGN cleavage products: FBN monomers that form the basis of the insoluble FBN scaffold and fibrinopeptides A and B. In addition, recent studies have shown that a greater range of soluble FGN cleavage products is generated by the action of thrombin on FGN. However, the characteristics and functions of these peptides remain mostly unknown.

5.1.1.2 Fibrinolysis and Fibrin Degradation Products

During fibrinolysis, plasmin cleaves FBN into a well characterised range of FBN degradation products that are distinct from those generated by thrombin (Walker and Nesheim, 1999; Liu *et al.,* 1986). These FBN degradation products comprise various peptides containing combinations of cross-linked E and D domains, including DD, DE, DDE, DDD and DDDD (diagram 5, page 47; Mosseson, 1990, for review).

Soluble FGN and polymerised FBN are equally good substrates for plasmin (Pandya *et al.*, 1985). In particular, residues Bβ1-42 of the Bβ-chain which contains an important site for the polymerisation of FBN (Hsieh, 1997) are exposed on the surface of the molecule (Pandya *et al.*, 1985). Plasmin can cleave peptides Bβ1-42 off the FGN Bβ-chain or peptide β15-42 off the FBN β-chain (Hurlet-Jensen *et al.*, 1983). However, the protease inhibitor α2-AP is found cross-linked into mature FBN clots and promotes the cleavage of peptide Bβ1-42 in FGN rather than peptide β15-42 in FBN (Takada *et al.*, 1986). Peptide Bβ1-42 is the first to be cleaved by plasmin, followed by peptide Bβ1-21, during the digestion of FGN (Koehn *et al.*, 1983). This suggests the presence of at least two plasmin degradation sites in the FGN Bβ-chain amino terminus. Moreover, the limited proteolysis of the Bβ-chain amino terminus is not accompanied by conformational changes of the FGN molecule (Pandya *et al.*, 1985).

Finally, other proteases produced mainly by inflammatory cells such as macrophages, neutrophils and mast cells can also degrade the FBN scaffold and cleave the FBN β-chain. The FGN β-chain amino terminus in particular encompasses several characterised cleavage sites for macrophage cathepsin D (Simon *et al.*, 1994) and neutrophil elastase (Bos *et al.*, 1995). Thus, these proteases may generate a range of cleavage products encompassing residues β15-42 through the proteolytic digestion of FBN and FGN.

5.1.2 The Cellular Effects of Fibrin, Fibrinogen and Fibrinogen-Derived Peptides

In addition to their roles during haemostasis, FGN, FBN and their derived peptides stimulate cellular functions *in vitro* and *in vivo* and they might stimulate fibroblast functions. These peptides are formed through the action of thrombin on FGN during blood clotting, as well as through the action of plasmin and other proteases during later fibrinolysis. In the following sections, the cellular effects of FGN and FBN cleavage products will be described.

5.1.2.1 Chemotaxis and Migration

Fibrinopeptides A and B are chemotactic for neutrophils, monocytes (Herrick *et al.*, 1999, for review), fibroblasts (Senior *et al.* 1986) and other cells. In addition, FGN Bβ-chain also has chemotactic properties for both inflammatory and mesenchymal cells (Skogen *et al.*, 1988). Indeed, peptides that mimic residues B1-14 (fibrinopeptide B) as well as residues β15-42 (revealed upon thrombin cleavage) promote chemotaxis more strongly than fibrinopeptide B alone

(Skogen *et al.*, 1988). This suggests that the Bβ-chain amino terminus contains at least two chemotactic domains.

Moreover, the insoluble FBN lattice provides a substrate for cells to migrate on and infiltrate the wound area. However, the presence of FBN into the wound space can also impair cell migration, depending on the relative amounts of FGN and thrombin present at the time of clotting as well as the degree of FBN cross-linking by factor XIIIa (Brown *et al.*, 1993). These parameters shape clots with different fibre structures and susceptibility to hydrolysis that allow cell invasion with various efficiency. FBN clots prepared with near normal concentrations of FGN and thrombin supported maximal macrophage migration (Ciano *et al.*, 1986) and extensive cross-linking of the FBN α-chains leads to even greater fibroblast, smooth muscle cell and macrophage migration (Ciano *et al.*, 1986; Brown *et al.*, 1993; Naito *et al.*, 1998).

Fibroblast migration out of damaged ECM into provisional FBN networks is facilitated by the deposition of fibronectin in their path which provides additional anchoring sites (Greiling*et al.* 1997). Factor XIIIa enhances this process further by cross-linking fibronectin to the FBN strands (Corbett *et al.*, 1997). Similarly, uncross-linked FBN is known to inhibit keratinocyte adherence and this effect can be reverted by the addition of fibronectin and factor XIIIa (Weiss *et al.*, 1998).

5.1.2.2 Adhesion and Spreading

Discrete regions of the FBN fibres mediate cell adherence and spreading to the FBN clot (Clark *et al.*, 1996, for review). FBN that lacks fibrinopeptide A or B promotes the adhesion of platelets (Rooney *et al.*, 1998), endothelial cells (Smith *et al.*, 1997) and fibroblasts (Gailit *et al.*, 1997; Asakura *et al.*, 1997) via the recognition of RGD sequences by cell surface integrins. Indeed, endothelial cell attachment to FBN lattices can be inhibited by the addition of soluble RGD-containing peptides (Chang *et al.*, 1995). In addition, des-AB FBN promotes fibroblast attachment by 30% over des-A FBN (Pardes *et al.*, 1995), suggesting that cleavage of fibrinopeptide B and the exposure of the β-chain amino terminus enhances further the effect of FBN on cell adhesion.

FBN also promotes endothelial cell and fibroblast spreading (Sporn *et al.*, 1995) and this only occurs onto FBN which lacks fibrinopeptide B (Francis *et al.*, 1993; Odrljin *et al.*, 1996). This appears to be mediated by the β-chain amino terminus that is revealed upon cleavage by thrombin (Bunce *et al.*, 1992; Hamagushi *et al.*, 1993; Francis *et al.*, 1993). Residues β15-42 are highly conserved between human and rat (Courtney *et al.*, 1994), suggesting a specific and

conserved role during homeostasis. Finally, the proteolytic removal of peptide β15-42 off the FBN β-chain amino terminus by plasmin during later fibrinolysis abolishes fibroblast and endothelial cell spreading onto FBN scaffolds (Hamagushi *et al.*, 1994). This suggests that the β-chain amino terminus is central to cell spreading on FBN lattices.

Other studies showed that soluble peptides released from the FGN Bβ-chain amino terminus during FBN deposition and fibrinolysis may cause endothelial cell activation, retraction, the release of von Willebrand factor and oedema formation (Rowland *et al.*, 1984; Cierniewski *et al.*, 1986; Ribes *et al.*, 1989). Their mechanism of action is not completely understood, but may involve residues β43-47 of FBN β-chain.

In summary, the FBN β-chain amino terminus in FBN scaffolds regulates cellular functions such as attachment, spreading and retraction. Furthermore, the presence of FBN and degradation products at sites of tissue injury is regulated by the coagulation and fibrinolytic cascades. Peptides originating from this region may contribute to tissue repair by promoting cellular invasion of healing tissues.

5.1.2.3 Platelet Aggregation

Peptides containing residues β15-42 prevent platelet aggregation (Chen *et al.*, 1988) by competing with FGN binding to cell surface glycoprotein IIb-IIIa (Savage and Ruggeri, 1991). This inhibitory effect is retained by shorter peptides containing the residues β19-42, but not by residues β15–18 or fibrinopeptide B only (residues 1-14). This may be explained by the ability of peptide containing residues β15-24 to interact with FBN and FGN molecules and inhibit FBN polymerisation (Pandya *et al.*, 1991). Thus, peptide β15-42 was suggested to prevent free association of platelets with blood FGN and the formation of platelet-rich clots.

5.1.2.4 Proliferation

FBN provides a substrate for cells to migrate into the wound space and regulates cell proliferation at sites of injury via residues β15-42 of the β-chain amino terminus (Sporn *et al.*, 1995). In addition, polymerised FBN promotes fibroblast and endothelial cell proliferation and this effect is mediated by residues β15-42 (Sporn *et al.*, 1995). A recent study suggested that basic fibroblast growth factor (bFGF) binds specifically to residues β15-42 (Sahni *et al.*, 1998). bFGF stimulates endothelial cell and mesenchymal cell proliferation and it is a particularly important

factor for angiogenesis (Choy *et al.,* 1996; Araki *et al.*, 1990). Thus, the mitogenic effect of FBN clots on endothelial cells can be partly explained by the presence of bFGF (Sahni *et al.,* 1999).

Finally, some FGN cleavage products generated by thrombin stimulate fibroblast (Gray *et al.,* 1995a) and mesothelial cell proliferation (Griffith *et al.,* 1994). It has been suggested that peptides in the supernatant of clots formed *in vitro* promote fibroblast proliferation independently from thrombin (Gray *et al.*, 1995a). The majority of this effect was attributed to peptides with a M_r between 200 and 300 kDa (Gray *et al.,* 1995a). In addition, studies with purified FGN chains showed that the FGN Aα– and Bβ–chains stimulated human fibroblast proliferation by up to 35% above controls (Gray *et al.*, 1993). A cell-surface form of calreticulin may play a role in the effect of FGN Bβ–chain in these cells (Gray *et al.*, 1995b).

5.1.2.5 Extracellular Matrix Production

As inflammatory cells, endothelial cells and fibroblasts invade the FBN clot, they change the ECM environment of the injured tissue. This affects the production and deposition of ECM. The topical application of FGN cleavage products to chick chorioallantoic membrane wounds stimulated angiogenesis and tissue fibrosis (Thompson *et al.,* 1986). In addition, FBN degradation products of less than 50 kDa M_r reproduce these effects *in vitro* and stimulate rapid *de novo* procollagen synthesis as well as prolyl hydroxylase activity, which is necessary for collagen deposition (Thompson *et al.,* 1986). Some studies showed that Fibroblast procollagen synthesis is also down-regulated by over three fold when the cells are grown on FBN matrices rather than tissue culture plastic (Pardes *et al.*, 1995). It was also suggested that fibrinopeptides A and B increase and decrease α1(I) procollagen mRNA levels, respectively (Pardes *et al.*, 1995). Others showed that skin fibroblasts increase their synthesis of type I and III collagens while increasing the ratio of collagen type III to type I when grown in artificial FBN matrices in the presence of thrombin (Michel and Harmand, 1990). Thus, there is some evidence that FBN lattices and fibrinopeptides modulate fibroblast procollagen synthesis.

Finally, the invasion of the FBN lattice and the deposition of collagen by fibroblasts modifies the clot's properties. Fibroblasts stimulate tPA production and fibrinolysis (Lorimier *et al.,* 1997) and promote FBN contraction, leading to a 90% reduction of the volume of FBN clots in two days (Tuan *et al.,* 1996). In contrast, newly deposited collagen strengthens FBN clots and slows down fibrinolysis (Mirshahi *et al.,* 1988; Mirshahi *et al.,* 1991).

Taken together, these data suggest that fibroblasts modify the characteristics of FBN clots whereas various FBN-derived moieties modulate fibroblast functions.

5.1.3 Summary and Aims of this Chapter

5.1.3.1 Summary

The biology of FGN, FBN and degradation products has been reviewed with a particular focus on their effects on cell functions. This has led to the following conclusions:

- Connective tissue and inflammatory cells are in direct contact with the FBN scaffold and in the immediate vicinity of FGN degradation products generated during coagulation.

- Insoluble FBN and soluble FGN cleavage products, including fibrinopeptides A and B. promote fibroblast migration, attachment, spreading and proliferation *in vitro* via discrete regions that are revealed upon cleavage with thrombin.

- Thrombin generates a great variety of FGN cleavage products. Some of these novel peptides stimulate fibroblast proliferation and this effect is mimicked *in vitro* by FGN Bβ-chain. However, the precise origin and function of these peptides remain unknown.

- The availability of the FBN β-chain amino terminus and residues β15-42 governs numerous effects of the FBN lattice, including fibroblast spreading and migration.

5.1.3.2 Specific Hypothesis of this Chapter

The hypothesis upon which the studies in this chapter are based is that:

> FGN-derived peptides comprising residues β15-42 of the FBN β–chain stimulate fibroblast proliferation and procollagen production.

5.1.3.3 Aims of this Chapter

To address this hypothesis, this chapter aims:

- To assess the effect of synthetic peptides mimicking the amino terminus of the FBNβ-chain on fibroblast DNA synthesis and proliferation *in vitro*.

- To determine the effect of FGN-derived peptides on fibroblast protein and procollagen production *in vitro*.

5.2 RESULTS

5.2.1 Effect of Fibrin Clot Supernatant on Fibroblast Proliferation

Figure 5.1 shows the mitogenic effect of fibrin clot supernatant on human foetal lung fibroblasts using the methylene blue dye binding assay after 48 hr incubation in the absence of serum. FBN clot supernatant stimulated fibroblast proliferation by up to 80% above control.

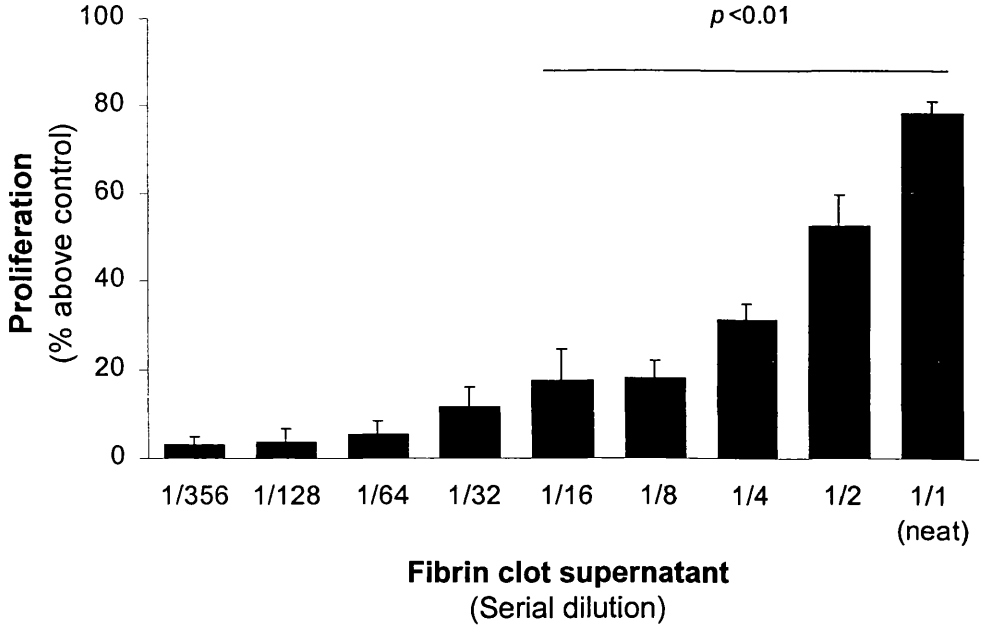


Figure 5.1 **Fibrin clot supernatant stimulates fibroblast proliferation**
Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., representative experiment with 6 replicates for each value.

Figure 5.2 shows that thrombin stimulated proliferation by up to 140% increase above control at 100 nM in the methylene blue dye binding assay and similar conditions to FBN clot supernatant. This stimulation was dose-dependent and significant at concentration greater than 1 nM. The EC_{50} was between 10 and 25 nM.

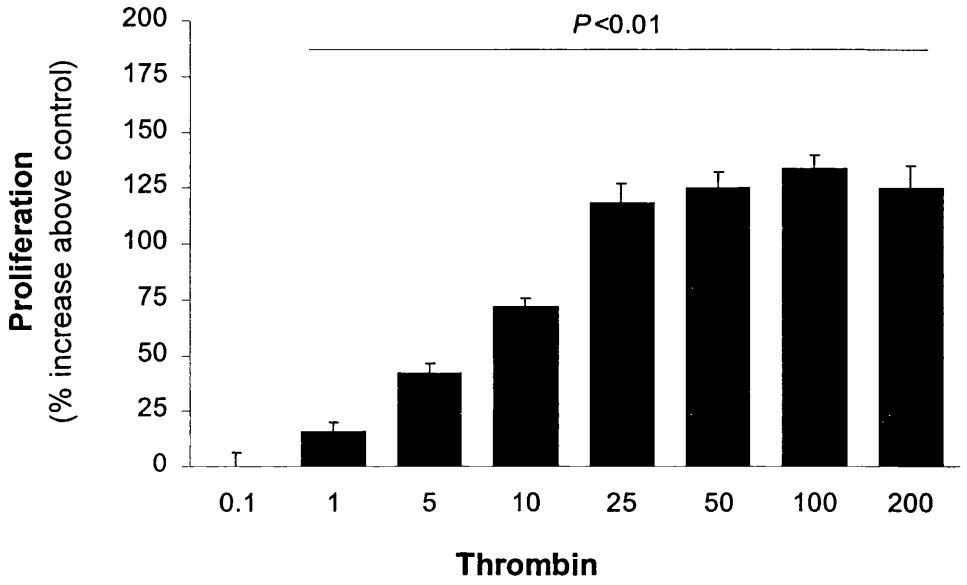


Figure 5.2 Thrombin stimulates fibroblast proliferation

Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., representative experiment with 6 replicates for each value.

To determine the contribution of thrombin and FGN cleavage products to the mitogenic effects of FBN clot supernatant, its effect on fibroblast proliferation was assessed in the presence and absence of leech hirudin, a potent and specific inhibitor of the proteolytic activity of thrombin.

Figure 5.3 shows that the mitogenic effects of FBN clot supernatant and that of thrombin alone were completely abolished by hirudin. In addition, there was no difference between the effects of FBN clot supernatant and thrombin alone at a concentration equivalent to that present in neat supernatant (6 nM).

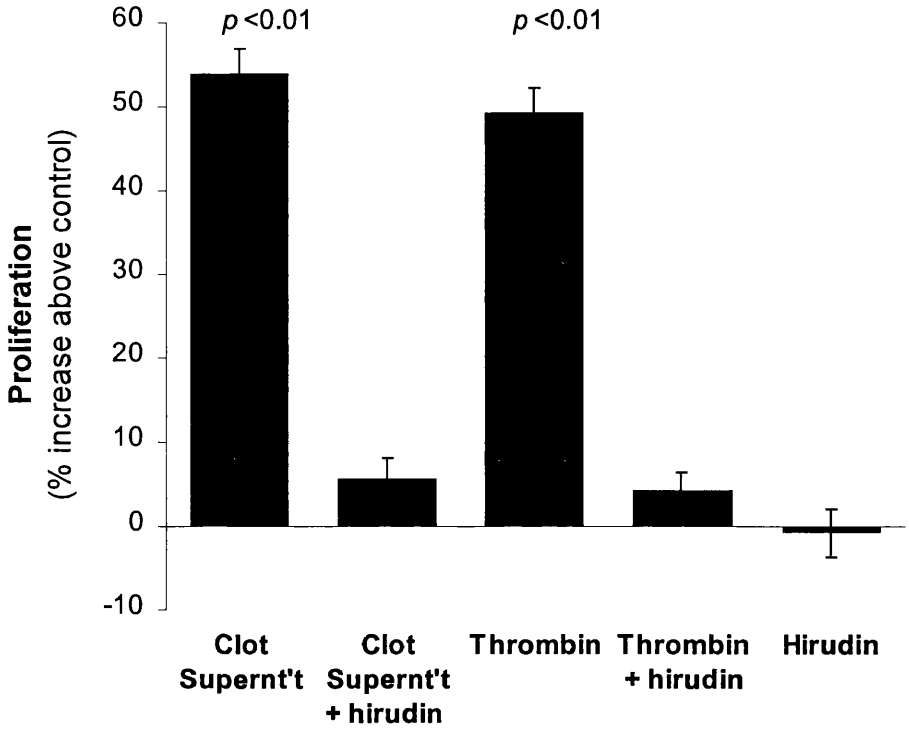


Figure 5.3 **Hirudin inhibits fibrin clot supernatant- and thrombin-stimulated fibroblast proliferation**

Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., representative experiment with 6 replicates for each value. FBN clot supernatant was used neat. Thrombin (6 nM) was used at the concentration present in FBN clot supernatant and native hirudin at 10 nM.

5.2.2 Effect of Fibrin Clot Supernatant on Fibroblast Procollagen Synthesis

To investigate further the profibrotic potential of FGN cleavage products, their ability to regulate other fibroblast functions was studied. The effect of FBN clot supernatant on human foetal lung fibroblast procollagen synthesis was assessed by measuring their hydroxyproline (hyp) production over 48 hr of incubation in the absence of serum.

Figure 5.4 shows that FBN clot supernatant (neat) stimulated fibroblast procollagen production by up to 62% above control. In comparison, the profibrotic growth factor TGF-β_1 (5 ng/ml) stimulated procollagen production by over 260% above controls. Thrombin alone, at the concentration used to generate the FBN clot supernatant *in vitro* (6 nM) stimulated a similar increase in procollagen production. Finally, the thrombin specific inhibitor PPACK completely abolished FBN clot supernatant- and thrombin-stimulated procollagen production.

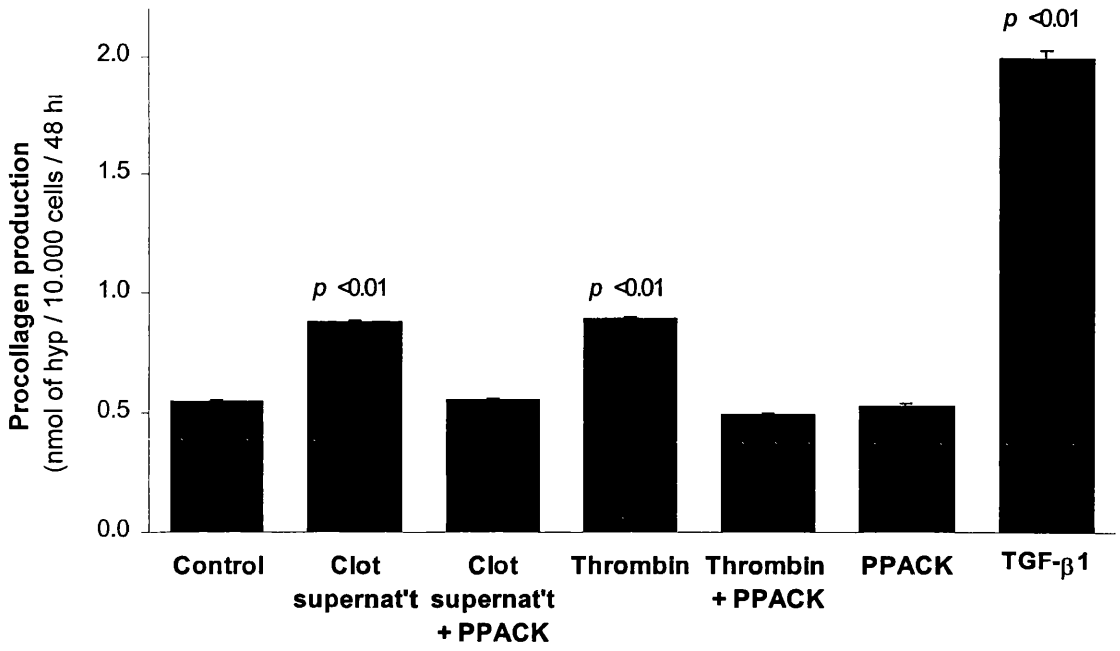


Figure 5.4 **PPACK inhibits thrombin- and fibrin clot supernatant-stimulated fibroblast procollagen production**

Hydroxyproline HPLC assay; expressed in nmol of hyp produced per 10.000 cells per 48 hr ± s.e.m., representative experiment with 6 replicates for each value. FBN clot supernatant is neat, thrombin (6 nM) is prepared in the same manner and to the same concentration. PPACK is used at 25 nM. *p* calculated against control.

5.2.3 Effect of Fibrinogen Bβ-chain and Fibrin Peptide β15-42 on Fibroblast Proliferation

5.2.3.1 Methylene Blue Dye Binding Assay

Effect of Fibrinogen Bβ-chain

Figure 5.5 and 5.6 show the mitogenic effect of FGN Bβ-chain on human foetal lung and adult lung fibroblasts. The assay and cell culture conditions were the same as those previously used by Gray and Colleagues (Gray *et al.,* 1992) and fibroblasts were incubated with FGN Bβ-chain for 48 hr in the absence of serum.

FGN Bβ-chain significantly stimulated human fibroblast proliferation in a dose-dependent fashion, at 0.6 10^{-7} M and higher concentrations. FGN Bβ-chain stimulated a maximal increase in proliferation at 5 10^{-7} M, by up to 35% above medium control in human foetal fibroblasts ($p<0.05$) and by up to 20% above medium control in human adult fibroblasts ($p<0.05$). The

mitogenic effect of FGN Bβ-chain was modest compared to the effect of 10% NCS, about 180% increase above control ($p<0.01$; data not shown).

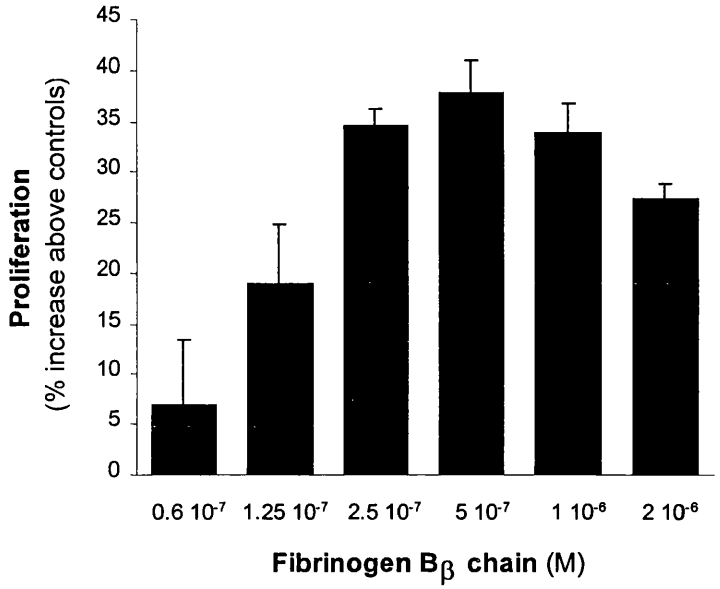


Figure 5.5 **FGN Bβ-chain stimulates human foetal lung fibroblast proliferation**
Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., mean of 6 independent experiments with 6 replicates for each value.

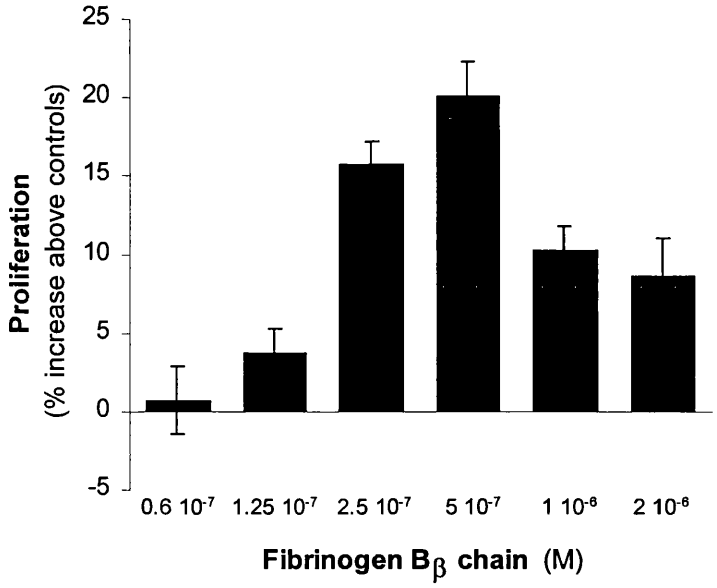


Figure 5.6 **FGN Bβ-chain stimulates human adult lung fibroblast proliferation**
Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., representative experiment with 6 replicates for each value.

Binding of Fibrinogen Bβ-chain to Tissue Culture Plastic

FGN Bβ-chain is highly negatively charged and its' ability to bind tissue culture plastic and bind methylene blue dye was investigated. 96 well tissue culture plates were pre-incubated with 5% NCS for 24 hr and incubated with purified FGN Bβ-chain (250 nM upwards) for 48 hr in the conditions used to perform proliferation assays with cells (37°C in the absence of serum).

Figure 5.7 shows that methylene blue binds to tissue culture plastic treated with FGN Bβ-chain at the concentrations used to stimulate fibroblast proliferationeith a maximal increase of up to 17 fold over untreated plastic ($p<0.05$).

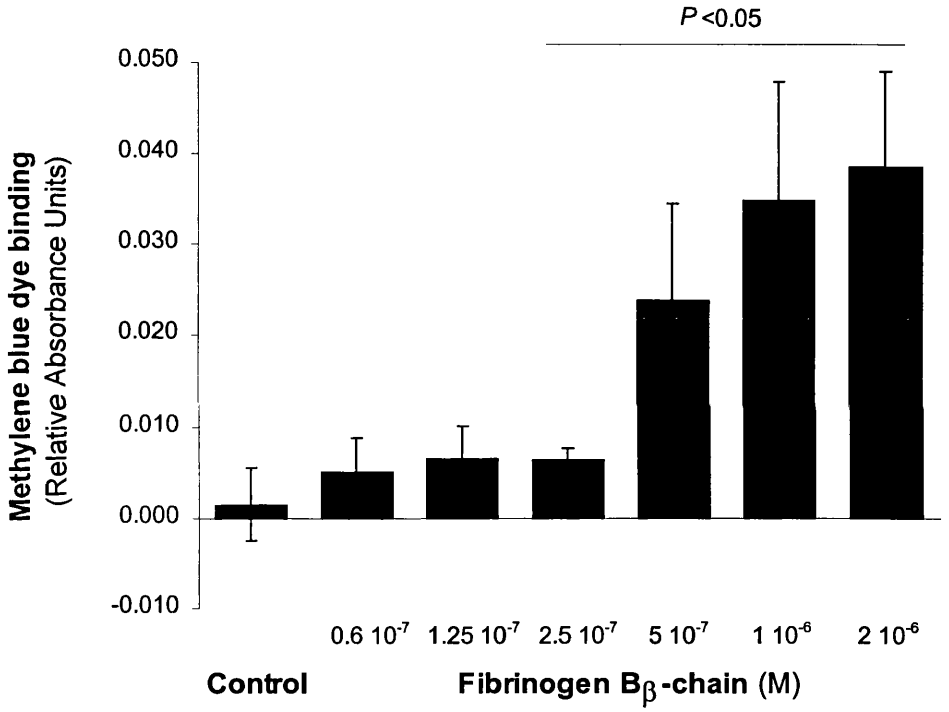


Figure 5.7 **FGN Bβ-chain increases methylene blue dye binding to plastic**
Methylene blue colorimetric assay; expressed in relative absorbance units, Representative experiment with 6 replicates for each value.

Effect of the Fibrinogen Bβ-chain Extraction Buffer

FGN Bβ-chain is prepared and stored in buffer containing 8 M urea and 125 to 170 mM sodium acetate (dilution 1/1; section 2.2.6.2). The Urea and sodium acetate concentrations are reduced by concentrating and rediluting FGN Bβ-chain in saline, however, some of this buffer remains present in the chain preparation when its effects on cell functions are assessed. The effect of the FGN Bβ-chain elution buffer alone on fibroblasts was studied in the methylene blue dye

binding assay. Figure 5.8 shows that the FGN Bβ-chain elution buffer causes up to 15% cell loss at concentrations comparable to that present on fibroblasts treated with FGN Bβ-chain.

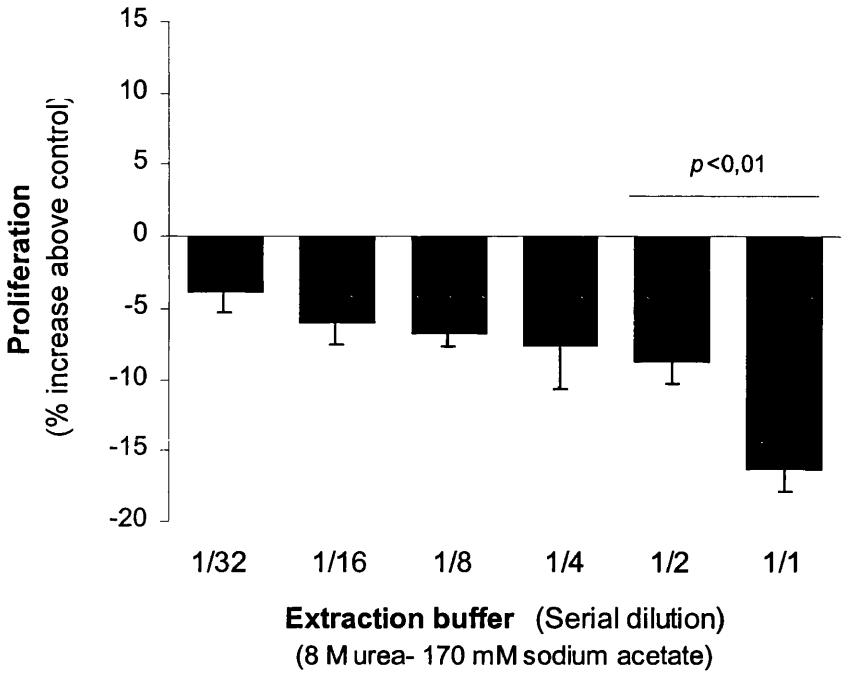


Figure 5.8 **Effect of the FGN Bβ-chain elution buffer on fibroblast proliferation**
Methylene blue colorimetric assay; expressed in absolute absorbance values, Representative experiment with 6 replicates for each value.

Effect of Fibrin Peptide β15-42 on Fibroblast Proliferation

It was hypothesised that soluble cleavage products comprising residues FBN β15- 42 regulate fibroblast proliferation and the effects on human foetal and adult lung fibroblast proliferation of synthetic peptides of the same sequence were evaluated. Culture conditions were similar to those described previously.

Figures 5.9 and 5.10 show that peptide β15-42 stimulated both human foetal and adult lung fibroblast proliferation and in a dose dependent fashion at concentrations superior to 10^{-8} M. The increase in proliferation reached up to 10% above medium control in human foetal fibroblasts at 10^{-6} M and by up to 12% above medium control in human adult fibroblasts at 10^{-7} M. However, the mitogenic effect of peptide β15-42 was about half of that of FGN Bβ-chain and remained modest compared to that of serum (10% NCS) that approximated 200% increase above control (data not shown).

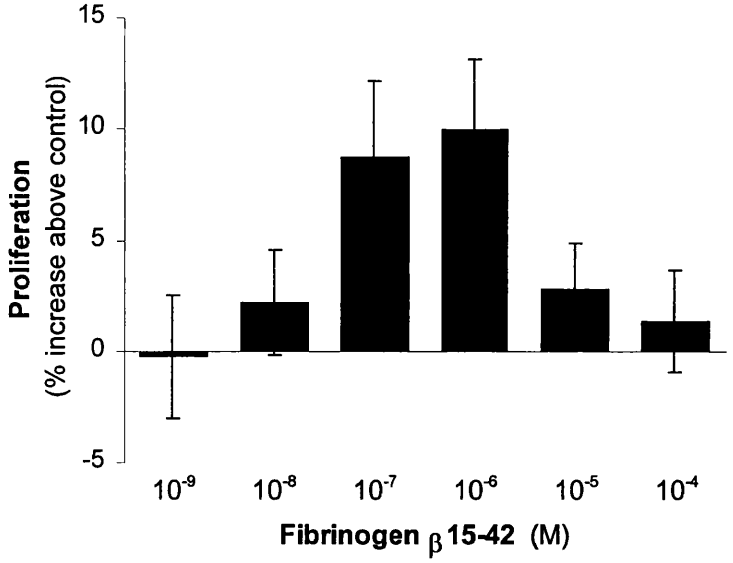


Figure 5.9 **FBN β15-42 peptide stimulates human foetal lung fibroblast proliferation**
Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., mean of 3 independent experiments with 6 replicates for each value.

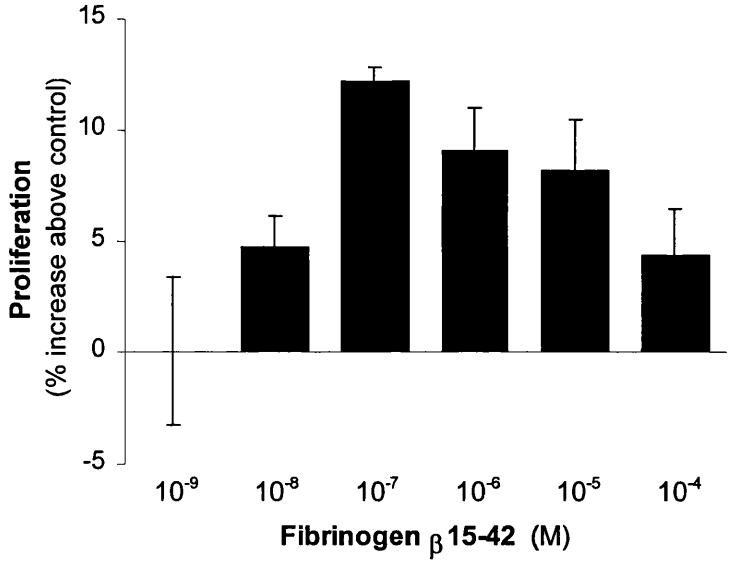


Figure 5.10 **FBN β15-42 peptide stimulates human adult lung fibroblast proliferation**
Methylene blue colorimetric assay; expressed in % increase above control ± s.e.m., representative experiment with 6 replicates for each value.

5.2.3.2 Direct Cell Counting

Figure 5.11 shows the time course of human foetal lung fibroblast proliferation in response to FGN Bβ-chain measured by direct cell counting after 24, 48 and 72 hr of incubation. FGN Bβ-chain did not stimulate increased proliferation at any time point. In contrast, the effect of serum was time-dependent and exponential, eliciting an increase of fibroblast proliferation of about 23% above control at 24 hr, 170% above control at 48 hr and 420% above control at 72 hr.

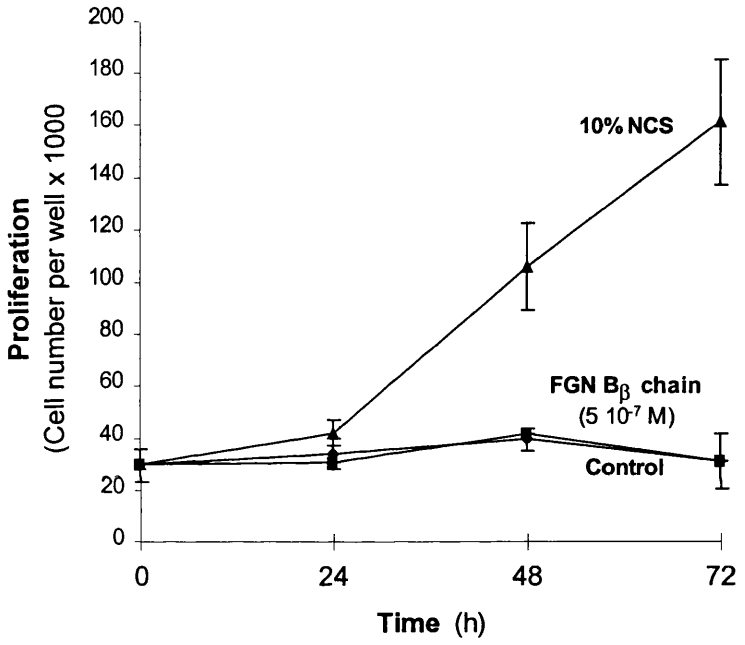


Figure 5.11 Time course of fibroblast proliferation in response to FGN Bβ-chain and neonatal calf serum

Direct cell counts;, expressed in cell number ± s.e.m., representative experiment with 4 replicates for each value.

5.2.4 Effects of Fibrinogen Bβ-Chain and Fibrin Peptide β15-42 on Fibroblast DNA Synthesis

The effect FGN Bβ-chain was assessed on human foetal lung fibroblast DNA synthesis using the ^{3}H-thymidine incorporation assay with two labelling methods. First, *de novo* DNA synthesis was pulse-labelled through incorporation of radioactive nucleotides between 16 hr and 20 hr of incubation with FGN Bβ-chain (figure 5.12). This method is particularly sensitive and suited to measure the effect of mitogens with immediate effects on cell DNA synthesis. Second,

de novo DNA synthesis was labelled continuously between 16 hr and 48 hr of incubation with the FGN chain (figure 5.13). This method was better suited to study potential mitogens with unknown time course of action.

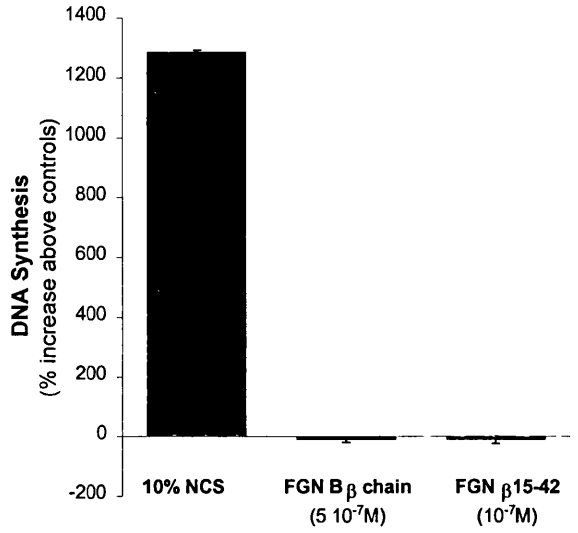


Figure 5.12 **FGN Bβ-chain and FGN β15-42 peptide do not stimulate fibroblast DNA synthesis (Pulse labelling)**

^{3}H-thymidine uptake assay (Pulse labelling from 16 hr to 20 hr after stimulation), expressed in % increase above control ± s.e.m., representative experiment with 6 replicates for each value.

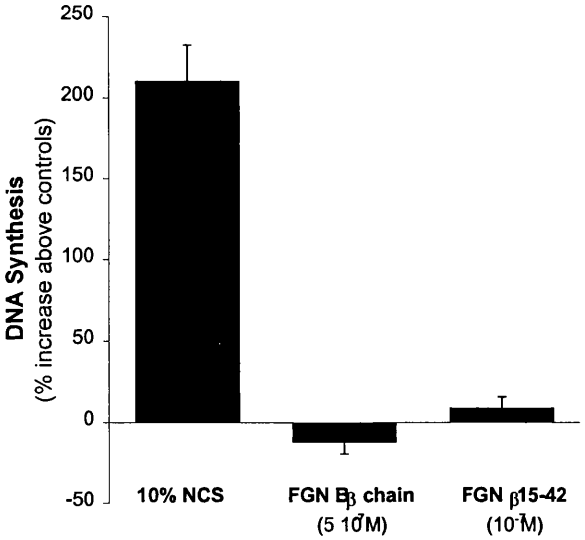


Figure 5.13 **FGN Bβ–chain and FGN β15-42 peptide do not stimulate fibroblast DNA synthesis (Continuous labelling)**

^{3}H-thymidine uptake assay (labelling from 16 hr to 48 hr after stimulation), expressed in % increase above control ± s.e.m., representative experiment with 6 replicates for each value.

Figures 5.12 and 5.13 show that FGN Bβ-chain did not stimulate an increase DNA synthesis using any of the labelling methods. The effect of peptide β15-42 was also assessed with these two labelling methods. Peptide β15-42 did not stimulate an increase DNA synthesis using any of the labelling methods. In contrast, serum (10% NCS) stimulated fibroblast proliferation significantly, eliciting an increase of about 1300% above control with the pulse labelling method and of 210% above control using the continuous labelling method

5.2.5 Effects of Fibrinogen Bβ-Chain and Fibrin Peptide β15-42 on Fibroblast Total Protein Synthesis

To further investigate the profibrotic potential of FGN Bβ-chain and peptide β15-42, their effects on human foetal lung fibroblast protein synthesis were investigated using the ^{3}H-phenylalanine incorporation assay over 48 hr of incubation and in the absence of serum. The peptides were evaluated at their maximal stimulatory concentrations in the methylene blue dye binding assay ($5\ 10^{-7}$ M).

Total protein produced by the fibroblasts was collected into a cell layer fraction and a cell supernatant fraction. This provided a crude evaluation of the effects of FGN peptides on total protein production, including that of non-collagenous ECM components. In addition, the measure of *de novo* protein synthesis provided an additional mean of assessing fibroblast mitogenesis since it should be associated with *de novo* protein synthesis.

Figures 5.14 and 5.15 show that neither FGN Bβ-chain nor FBN peptide β15-42 upregulated *de novo* protein synthesis, whether incorporated in the cell layer or secreted in the cell supernatant.

5.2.6 Effect of Fibrinogen Bβ-Chain on Fibroblast Procollagen Synthesis

The effect of FGN Bβ-chain was assessed on human foetal lung fibroblast procollagen production in serum free conditions for 48 hr, in the presence of ascorbate and proline according to a published method (Campa *et al.*, 1990).

Figure 5.16 shows that FGN Bβ-chain did not stimulate fibroblast procollagen production. In contrast, TGF-β_1 stimulated fibroblast procollagen synthesis by over 150% above control.

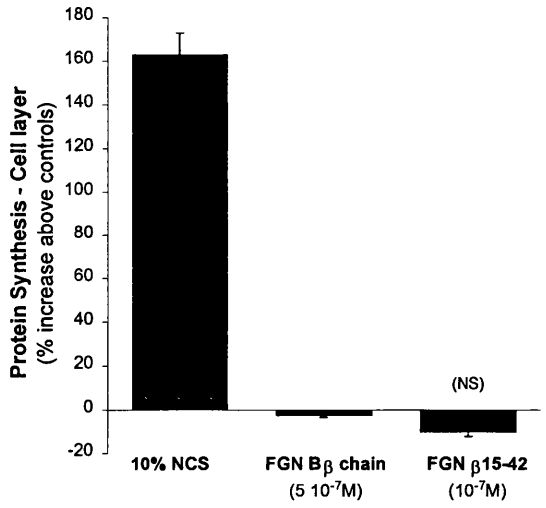


Figure 5.14 **FGN Bβ–chain and FGN β15-42 peptide do not stimulate the *de novo* synthesis of proteins incorporated into the fibroblast layer**

^{3}H-phenylalanine intake assay, expressed in % increase above control ± s.e.m., representative experiment with 4 replicates for each value.

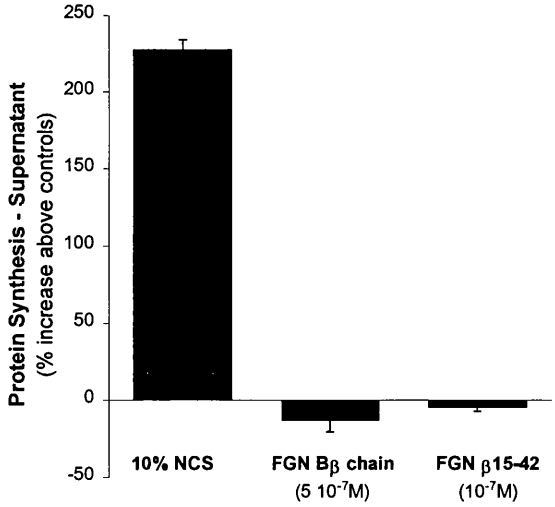


Figure 5.15 **FGN Bβ–chain and FGN β15-42 peptide do not stimulate the *de novo* synthesis of proteins secreted in the supernatant of fibroblasts in culture**

^{3}H-phenylalanine intake assay, expressed in % increase above control ± s.e.m., representative experiment with 4 replicates for each value.

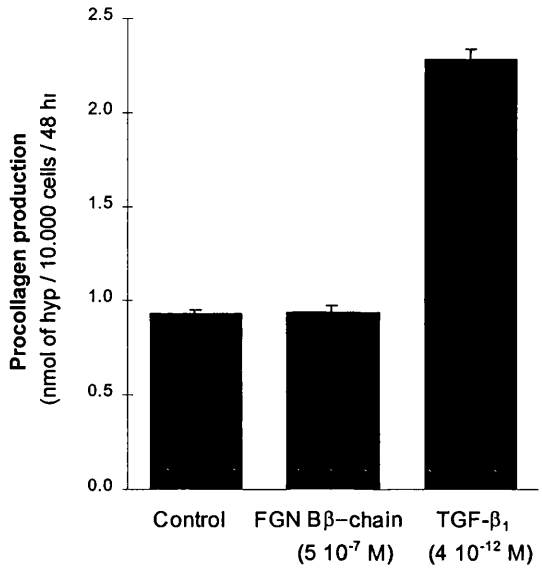


Figure 5.16 FGN Bβ–chain does not stimulate fibroblast procollagen production
Hydroxyproline HPLC assay; expressed in nmol of hydroxyproline produced per 10.000 cells per 48 hr ± s.e.m., representative experiment with 6 replicates for each value.

5.3 DISCUSSION

This section investigated the effects of FGN cleavage products extracted from FBN clots formed *in vitro*, purified FGN Bβ-chain and synthetic peptides mimicking the amino terminus of the FBN β-chain on fibroblast proliferation and protein production.

5.3.1 EFFECTS OF FIBRIN CLOT SUPERNATANT ON FIBROBLAST PROLIFERATION AND PROCOLLAGEN SYNTHESIS

Fibroblast Proliferation

Previous studies reported the presence of a broad range of FGN-derived moieties in the supernatant of FBN clots formed *in vitro*, some of which displayed mitogenic effects on fibroblasts (Gray *et al.*, 1990; Gray *et al.*, 1993; Gray *et al.*, 1995a) and mesothelial cells (Griffith *et al.*, 1994). The effect of FBN clot supernatant on human foetal lung fibroblast proliferation was evaluated with the methylene blue dye binding assay. My initial data were consistent with previous observations and showed that FBN clot supernatant stimulates fibroblast proliferation (Gray *et al.*, 1995a). This also confirmed that thrombin alone stimulates human foetal lung

fibroblast proliferation (Dawes *et al.*, 1993). In addition, the specific thrombin catalytic site inhibitor hirudin completely blocked FBN clot supernatant- and thrombin-stimulated fibroblast proliferation. Taken together, these data show that the mitogenic effect of FBN clot supernatant is mediated by the proteolytic activity of thrombin and that FGN cleavage products present in FBN clot supernatant have no effect on fibroblast proliferation.

Furthermore, thrombin present in FBN clot supernatant or alone at the concentration present in supernatants (6 nM) had similar effects on fibroblast proliferation. This suggests that thrombin retains its mitogenic potential after cleaving FGN into FBN*in vitro* and that its' activity can be virtually completely recovered in the soluble fraction of FBN clots by decantation.

Fibroblast Procollagen Production

FBN clot supernatant (neat) stimulated an increase in fibroblast procollagen production. This effect was equivalent to that of thrombin alone at the same concentration (6 nM). These effects of FBN clot supernatant and thrombin were completely inhibited by the specific thrombin inhibitor PPACK. These data support the observation that thrombin stimulates fibroblast procollagen production (Chambers *et al.*, 1998). The results also imply that the effect of FBN clot supernatant is entirely mediated by thrombin and soluble FGN cleavage products present in FBN clot supernatant have no effect on fibroblast procollagen production. Finally, the mitogenic effect of thrombin on fibroblasts is not affected by previous cleavage of FGN into FBN *in vitro*.

Summary

The results obtained were consistent with previous reports showing that FBN clot supernatant enhances fibroblast proliferation. Furthermore, I showed that FBN clot supernatant also promotes fibroblast procollagen production. However, data obtained with specific thrombin inhibitors show that these effects are due to thrombin alone and not FGN-derived moieties.

5.3.2 Effects of Fibrinogen Bβ-chain Peptides on Fibroblast Proliferation and Procollagen Synthesis

Fibrinogen Bβ-chain and fibroblast proliferation

The mitogenic effects of FGN Bβ-chain on the proliferation of our human foetal lung and human adult lung fibroblasts was measured with the methylene blue dye binding assay. The data obtained is consistent with previous observations (Gray *et al.*, 1993; Gray *et al.*, 1995b) that show

that FGN Bβ-chain stimulates methylene blue dye binding on fibroblasts in a dose-dependent fashion with a maximum of about 35% above medium control at 5.10^{-7} M.

More sensitive assays than methylene blue dye binding assay were also employed, including direct cell counting and ^{3}H-thymidine uptake. The ^{3}H-phenylalanine incorporation and was also used to determine *de novo* protein synthesis. For easy comparison of the results between assays, the experiments were performed in parallel and in similar conditions. When the mitogenic effect of FGN Bβ-chain was evaluated via direct cell counting, no increase of fibroblast proliferation was observed after 24 hr, 48 hr or 72 hr of incubation. Furthermore, FGN Bβ-chain did not stimulate *de novo* fibroblast DNA synthesis using either *pulse* or *continuous* labelling methods. Finally, FGN Bβ-chain did not stimulate the *de novo* synthesis of proteins incorporated in the cell layer or released from fibroblasts. These results do not correlate with my data as well as that of others that were obtained with the methylene blue dye binding assay and suggest a mild increase of fibroblast proliferation (Gray *et al.,* 1993; Gray *et al.*, 1995a).

Collectively, these data suggest that the increase in methylene blue dye binding stimulated by FGN Bβ-chain does not reflect increased cell number. Furthermore, three independent methods of assessing proliferation showed that this peptide does not stimulate fibroblasts.

Limitations of the Methylene Blue Dye Binding Assay

FGN is a highly negatively charged molecule and its' ability to bind in a non-specific fashion to a great variety of substrates, including tissue culture plastic, is well recognised (Mosseson *et al.*, 1989, for review). When FGN Bβ-chain is incubated on bare tissue culture plastic in the exact conditions used to assess fibroblast proliferation, it promoted methylene blue dye binding. This effect may be due to the adherence of FGN Bβ-chain to tissue culture plastic and subsequently to the binding of dye to the chain's negative charges. The increase in absorbance produced was comparable to that attributed to fibroblast proliferation in response to FGN Bβ-chain. It is concluded that the mitogenic potential of micromolar concentrations of negatively charged proteins such as FGN chains can not be assessed in the methylene blue dye binding assay. The maximal concentration of FGN chains that can be used without increasing dye binding non-specifically is 125 nM. Thus, the increase in methylene blue dye binding promoted by FGN Bβ-chain in our study and others where this assay was used (Gray *et al.*, 1990; Gray *et al.*, 1995a) may result from the binding of FGN Bβ-chain to plastic rather than cell proliferation.

In addition, the buffer in which FGN Bβ-chain is prepared inhibited fibroblast proliferation at concentrations equivalent to that present in preparations of FGN Bβ-chain of 1 μM

and upwards. Thus, FGN chains should only be utilised in the methylene blue dye binding assay at concentrations below 1 μM without further purification.

Taken together, the effects of FGN Bβ-chain on methylene blue dye binding to plastic and the inhibitory effects of its elution buffer on fibroblast proliferation suggest that data obtained in this assay in response to FGN Bβ-chain are not a valid representation of fibroblast proliferation. Furthermore, data obtained with the *de novo* DNA and protein synthesis assays as well as with direct cell counts suggest that FGN Bβ-chain does not stimulate fibroblast proliferation.

Fibrinogen Peptide β15-42 and Fibroblast Proliferation

The effects of peptide β15-42 on fibroblast proliferation were studied using the methylene blue dye binding assay, *de novo* DNA and protein synthesis assays. The data obtained show that high concentrations of FGN peptide β15-42 (10^{-8} to 10^{-5} M) stimulated a very modest increase in human foetal and adult lung fibroblast proliferation, reaching up to 10 to 15% increase above control in either cell type. However, FGN peptide β15-42 at the maximal mitogenic concentration in the methylene blue assay (10^{-7} M) did not stimulate *de novo* fibroblast DNA or protein synthesis, whether incorporated into the cell layer or secreted into the cell supernatant. These results suggest that soluble peptides comprising residues β15-42 that are generated during FBN deposition and lysis have no effects on fibroblast proliferation and protein production.

Fibrinogen Bβ-chain and Fibroblast Procollagen Production

The effect of FGN Bβ-chain on fibroblast procollagen production was studied at the maximal stimulatory concentration in the methylene blue assay (5.10^{-7} M). FGN Bβ-chain did not stimulate fibroblast procollagen production whereas TGF-β1 (5 ng/ml) stimulated an increase of about 165% above control. This suggests that FGN Bβ-chain does not regulate procollagen production by fibroblasts. This is consistent with previous reports showing that FGN, in contrast with FBN and FGN-derived peptides, does not affect other fibroblast functions such as chemotaxis, migration, proliferation or ECM production.

Summary

Using several complementary methods, this study showed that FGN Bβ-chain did not stimulate fibroblast proliferation, total protein and procollagen production in human foetal and adult lung fibroblasts. In contrast, the serine protease thrombin was a very potent mitogen for fibroblasts that remained fully active after converting FGN into FBN *in vitro*.

CHAPTER 6

CONCLUSION

This thesis investigated the effects of coagulation cascade peptides that are generated in the extrinsic coagulation pathway on fibroblast proliferation. It lead to several novel findings in two main areas:

THE CELLULAR EFFECTS OF COAGULATION CASCADE PEPTIDES

- FXa is a potent mitogen for fibroblasts and its' effect is similar to thrombin.
- FXa stimulates PDGF production and autocrine stimulation in fibroblasts.
- Factors VIIa and IXa have little effects on fibroblast proliferation.
- Soluble FGN cleavage products have no effects on fibroblast proliferation and procollagen production.

A NOVEL DUAL RECEPTOR SYSTEM MEDIATES THE MITOGENIC EFFECT OF FACTOR Xa ON FIBROBLASTS

- FXa binds to EPR-1 to stimulate fibroblast proliferation.
- FXa stimulates fibroblast proliferation via Gαi protein activation and cytosolic Ca^{2+} mobilisation.
- FXa activates PAR-1 to stimulate fibroblast proliferation.

The implications of these findings will be discussed in turn with respect to: a) Our understanding of tissue repair mechanisms, b) possible therapeutic applications that may arise from this work and c) experiments to further research in this field.

6.1 CELLULAR EFFECTS OF COAGULATION CASCADE PEPTIDES

Two groups of coagulation cascade peptides have been studied in this thesis: Serine proteases of the extrinsic and common coagulation pathways (chapter 3 and 4) and soluble FGN

cleavage products of thrombin (chapter 5). The effects of coagulation cascade peptides on fibroblasts including those described in this thesis are summarised in table 6 (page 183).

6.1.1 Effects of Coagulation Cascade Peptides on Fibroblasts

Fibrin and Fibrinogen Cleavage Peptides

The mitogenic potentials of three types of FGN-related peptides have been assessed in this thesis: FGN cleavage products present in the supernatant of FBN clots generated *in vitro*, purified FGN Bβ-chain and synthetic peptides mimicking the amino terminus of the FBN β-chain that is revealed upon cleavage by thrombin. Soluble FGN-derived peptides displayed little or no effect on fibroblast DNA synthesis and proliferation *in vitro*. Furthermore, none of the peptides affected fibroblast procollagen or total protein production *in vitro*.

However, it is known that some peptides generated through the action of thrombin on FGN affect fibroblast functions. First, fibrinopeptides A and B which are cleaved off the Aα- and Bβ-chains by thrombin, are chemotactic to fibroblasts (Senior *et al.*, 1986). Second, FBN provides a scaffold for cells to attach onto and promotes fibroblast spreading and migration at sites of tissue injury (Clark, 1996, for review). In addition, fibroblasts have increased rates of proliferation (Sporn *et al.*, 1995) and procollagen production when growing on FBN matrices (Michel and Harmand, 1990). Thus, FGN cleavage products modulate various fibroblast functions.

A great number of these effects of FBN on fibroblasts seem to be regulated via discrete regions of FGN, including residues β15-42 of the FBN β-chain amino terminus which promotes fibroblast spreading and proliferation (Francis *et al.*, 1993). Furthermore, the presence of residues β15-42 at sites of injury is temporally and locally regulated. They appear during blood coagulation through the cleavage of the FBN β-chain by thrombin and disappear during fibrinolysis via the action of a variety of proteases (section 5.1.2).

In summary, the cleavage of FGN into FBN by thrombin results in the generation of soluble chemoattractants and an insoluble substratum that regulates fibroblast adhesion and spreading. This study shows that the soluble products arising from FGN cleavage by thrombin do not promote fibroblast proliferation or procollagen production.

Coagulation Cascade Proteases

This study showed that fXa and thrombin are the most potent fibroblast mitogens *in vitro* that are activated through the extrinsic and common coagulation pathways. The kinetics and

magnitude of their mitogenic effects are very similar and compared to that of optimal concentrations of PDGF-AB. In contrast, factor VIIa and IXa had little effects.

However, factor VIIa has recently been shown to stimulate vascular endothelial growth factor production by fibroblasts (Ollivier *et al.,* 1998; Abe *et al.,* 1999) and this factor may contribute to endothelial growth and angiogenesis. Furthermore, factor VIIa has significant mitogenic effects on fibroblasts at concentrations greater than that of its' corresponding zymogen in normal plasma. This suggests that factor VIIa might contribute to connective tissue deposition in conditions associated with excessive and recurrent activation of the coagulation cascade.

The blood-borne precursor factor X had little effect on fibroblasts and the mitogenic effects of fXa depend upon its' catalytic activity (Coughlin, 1993; McNamara *et al.,* 1993). This is similar to the mode of action of thrombin. Moreover, the observation that thrombin remains in solution and stimulates fibroblast proliferation after cleavage of FGN into FBN *in vitro* suggests that its' procoagulant and cellular functions are not mutually exclusive but can occur simultaneously. This implies that thrombin gains its' mitogenic potential upon activation during blood coagulation, similarly to thrombin. Thus blood coagulation mediates normal haemostasis and the generation of fibroblast mitogens.

It can be further speculated that the mitogenic potential of fXa and thrombin for fibroblasts may be regulated in the same manner as their procoagulant activity by the comprehensive array of serine protease inhibitors present in plasma or secreted by tissue and inflammatory cells (section 1.1.2.1).

These data suggest that the activation of the coagulation cascade results in the generation of at least two fibroblast mitogens. Thus it is possible that the haemostatic and mitogenic effects of coagulation cascade occur concurrently, via similar mechanisms and may be inseparable.

Other Coagulation Cascade Peptides

In addition to peptides activated in the extrinsic and common coagulation pathways, other coagulation cascade peptides have been suggested to stimulate mitogenesis.

The anticoagulant factor activated protein S is vitamin K-dependent factor similarly to factor X and prothrombin. Activated protein S stimulates smooth muscle cell proliferation via its' four EGF repeats (Gasic *et al.,* 1992). Similarly, factor XIIa promotes smooth muscle, endothelial and type II lung cell proliferation (Gordon *et al.,* 1996) via its' EGF repeats (Gordon *et al.,* 1996). These mechanisms of action are different that of fXa and thrombin. However, the effects of protein S and factor XIIa on fibroblasts are still unknown.

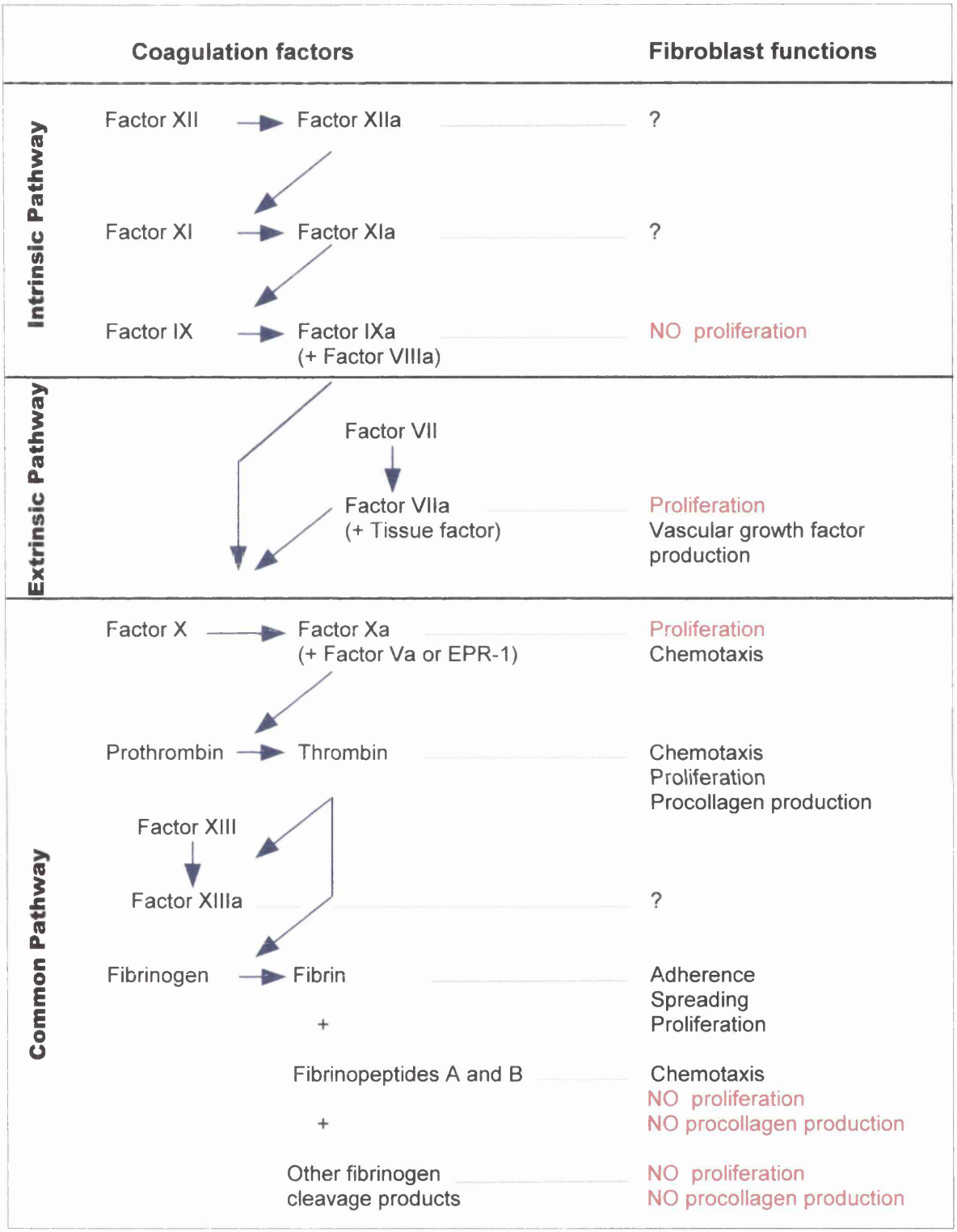


Table 6 **Effects of coagulation cascade peptides on fibroblasts**

This table summarises the principal effects of coagulation cascade peptides on fibroblasts. The effects highlighted in red are observations from this study. Other effects of coagulation cascade peptides on fibroblasts have been reviewed in this thesis (chapter 1 and introductions to chapter 3, 4 and 5). Oblique arrows denote coagulation factor activation by limited proteolysis.

In summary, fXa was shown for the first time to be mitogenic for fibroblasts. In addition, fXa and thrombin, but not all coagulation factors, stimulate fibroblast proliferation. Thus, the majority of the mitogenic effects of the extrinsic and common coagulation pathways appears to reside in two proteases.

Fibrinolytic Proteases

Although urokinase-type plasminogen activator (uPA) is not strictly a coagulation factor, it is one of the main mediators of fibrinolysis and promotes fibroblast proliferation (De Petro *et al.*, 1994; Shetty *et al.*, 1996). This effect of uPA is promoted by binding to the uPA receptor (uPAR; Tkachuk *et al.*, 1996, for review) and at least part of this effect is mediated by the release of bFGF from the ECM (Vlodavsky *et al.*, 1990, for review). In addition, thrombin stimulates uPA expression in human fibroblasts *in vitro* (Hayakawa *et al.*, 1995). This suggests that thrombin may lead to further fibroblast proliferation via this mechanism. However, the production of uPA by tissue cells occurs mainly in response to FBN.

PDGF Stimulation

This thesis shows that the mitogenic effects of thrombin and fXa on fibroblasts are mediated by autocrine stimulation of PDGF. This mechanism is consistent with observations in smooth muscle cells (Ko *et al.*, 1996; section 3.2.3.5) and similar to that of other proteases, including thrombin that stimulates PDGF-AA production and PDGF receptorα subunit expression in human fibroblasts (Ohba *et al.*, 1994; Ohba *et al.*, 1996).

Thrombin also promotes platelet degranulation and causes the release of a great variety of cytokines, including PDGF and TGF-β1 (Kaplan *et al.*, 1979; Assoian and Sporn, 1986). The three isoforms of PDGF (AA, AB and BB) are present in platelet granules (Hart *et al.* 1990; Soma *et al.*, 1992). This provide thrombin with indirect means of stimulating fibroblast proliferation (Fretto *et al.*, 1993; Bonner *et al.*, 1991; Clark *et al.*, 1993). The generation of active thrombin by fXa may contribute to this mechanism.

PDGF-AB appears to be the only isoform that up-regulates procollagen gene expression and collagen deposition during tissue repair (Lepisto *et al.*, 1996). PDGF-AA and -BB have been suggested to have little or no effect on procollagen production (Clark *et al.*, 1993; Lepisto *et al.*, 1995). Thus, PDGF autocrine stimulation of fibroblasts *in vitro* is thought to be mostly relevant to proliferation rather than matrix production.

Finally, PDGF is known to stimulate other growth factors such as TGF-β1 in fibroblasts (Pierce *et al.,* 1989). These growth factors can subsequently up-regulate expression of their own receptors as well as that of other cytokines (Ishikawa *et al.,* 1990; Ichiki *et al.,* 1995), thereby potentiating their effects. The production of PDGF by fibroblasts in response to thrombin or fXa may trigger the development of a complex growth factor network with an array of consequences for the surrounding tissue.

Furthermore, *in vitro* evidence suggests that growth factors such as PDGF-AB and TGF-β1 (section 1.1.1) up-regulate PAR-1 expression in mesenchymal cells (Schini-Kerth *et al.*, 1997). This may lead to elevated PAR-1 expression during connective tissue formation and enhance the effects of thrombin and fXa.

6.1.2 Role of Coagulation Cascade Peptides in Tissue Repair and Fibrosis

This thesis showed that at least two coagulation cascade proteases are mitogenic to fibroblasts *in vitro*, including fXa. This has implications on our understanding of connective tissue formation during tissue repair and fibrosis.

6.1.2.1 Tissue Repair

It is known that the topical administration of thrombin or synthetic peptides that mimic its sequence to dermal incisional wounds enhances tissue vascularisation, tensile strength and collagen type I accumulation by about 40% (Carney *et al.*, 1992; Warren *et al.,* 1992). Furthermore, thrombin peptides accelerate wound closure in full thickness excisional wound healing models (Pernia *et al.,* 1990; Warren *et al.*, 1992) and the local administration of thrombin in experimental models of impaired wound healing due to radiotherapy promotes normal wound healing (Cromack *et al.*, 1992). Taken together with evidence that thrombin promotes fibroblast proliferation and procollagen production *in vitro* (Connolly *et al.*, 1996; Chambers *et al.,* 1998), these observations suggest that thrombin contributes to tissue repair.

Little is known of the effects of fXa on tissue repair and similar experiments could be done *in vivo* to demonstrate the profibrotic potential of fXa. On the basis of results in this thesis that show that the mitogenic effects of fXa are equivalent to thrombin *in vitro* and that fXa activates PAR-1, fXa might be expected to stimulate tissue repair similarly to thrombin.

6.1.2.2 Tissue Fibrosis

Aberrations in the activation or inhibition mechanisms of blood coagulation may cause active coagulation cascade peptides to persist in the tissue and cause pathological conditions (section 1.1.2.1). Furthermore, there is increasing evidence that the disruption of procoagulant and anticoagulant activities at sites of tissue injury is a feature common to fibrotic diseases. The lungs contain an extensive microvascular vascular bed and the repetitive exposure to airborne particles makes them particularly susceptible to injury and tissue fibrosis.

Clinical Evidence

FBN deposition in the is a hallmark of tissue fibrosis in response to injury and this is particularly relevant to the lung (Chapman *et al.,* 1986; Idell, 1994 for review). Lung fibrosis is characterised by abnormal fibroblast proliferation and excessive collagen deposition (section 1.1.1.2) and recent studies have shown that there is a correlation between procollagen processing in the lungs and mortality in acute lung fibrosis such as ARDS (Meduri *et al.*, 1998). In addition, increased levels of tissue factor and associated factor VIIa can be measured in the bronchoalveolar lavage fluid from patients with ARDS (Idell *et al.,* 1987a; Idell *et al.,* 1989c), suggesting persistent and excessive activation of the extrinsic coagulation cascade in their lungs. Increased FBN deposition has also been suggested to be a feature of neonatal respiratory distress syndrome and correlate with the severity of the symptoms (Schmidt, 1994 for review).

In addition, abnormal activation of the coagulation cascade is a feature of chronic fibrotic lung diseases. Indeed, FBN *hyaline membranes* and persistent FBN deposition are used for the diagnosis of interstitial lung diseases (Seeger *et al.,* 1993, for review). In addition, increased levels of fibrinopeptide A and tissue factor (Nakstad *et al.,* 1990) as well as thrombin and activated protein C (Kobayashi *et al.*, 1998) have been shown to be elevated in idiopathic pulmonary fibrosis and sarcoidosis. This reflects a profound imbalance in regulatory mechanisms of the coagulation cascade in chronic fibrosis.

Similar findings have been made in pulmonary fibrosis associated with vasculitic diseases, including systemic sclerosis (Ames *et al.,* 1997). Moreover, thrombin is a major fibroblast mitogen present in the bronchoalveolar lavage fluid of patients with lung fibrosis associated with systemic sclerosis (Hernandez-Rodriguez *et al.,* 1995). Sustained levels of prothrombin activation and FBN deposition have also been measured in interstitial lung disease associated with

rheumatoid arthritis where levels of fibrinopeptide A, tissue factor and associated factor VIIa are elevated (Idell *et al.*, 1989b).

Thus excessive collagen deposition and activation of the coagulation cascade occur concurrently in fibrotic diseases of the lung. These events may be linked and coagulation cascade proteases might contribute to tissue fibrosis.

Evidence in Animal Models

Animal models have been employed to reproduce excessive collagen deposition experimentally in the lung (Shahzeidi *et al.*, 1994) and study the role of the coagulation cascade in the development of fibrotic lung diseases. High levels of tissue factor and factor VIIa activity have been noted in bronchoalveolar lavage fluid in bleomycin-induced lung fibrosis (Idell *et al.*, 1987b; Idell *et al.*, 1989a). Tissue factor/factor VIIa activity correlated with prominent alveolar FBN deposition and decreased anticoagulant activity in alveolar lining fluids. In bleomycin-induced pulmonary fibrosis, levels of procoagulant activity increased within four hr after challenge and remained elevated for over two weeks, suggesting chronic activation of the extrinsic pathway (Idell *et al.*, 1989a). Heparin treatment in animal models of lung fibrosis improves gas exchange and the fibrotic outcome (Abubakar *et al.*, 1998), consistent with a role of the coagulation cascade in the fibroproliferative process.

Genetically Modified Animals

Prothrombin-deficient mice have been generated by several groups (Xue *et al.*, 1998; Sun *et al.*, 1998). About half of these animals die *in utero* around day 10 to 11 of development with severe vasculature defects and haemorrhage. The remaining embryos do not survive more than a day after birth and also dye from the same cause (Xue *et al.*, 1998; Sun *et al.*, 1998). The study of tissue repair and fibrosis has not been possible in these animals. This suggests that prothrombin and possibly the generation of thrombin is central to preserve vascular integrity during morphogenesis. In addition, there is no information on fXa-deficient animals. Taking into account the prothrombin deficiency phenotype, one might expect a highly penetrant lethal phenotype during embryogenesis for fXa-deficient animals.

Summary

Excessive and recurrent activation of the coagulation cascade is observed during fibrotic lung diseases and in experimental animal models. A relationship may exist between fibrosis and

the extravasation of coagulation cascade peptides and excessive levels of coagulation cascade peptides may form a milieu favourable to excessive mesenchymal cell proliferation and ECM deposition, leading to loss of organ function and morbidity. It is possible to extrapolate that fXa might exacerbate development of tissue fibrosis.

6.2 A NOVEL DUAL RECEPTOR SYSTEM MEDIATES THE MITOGENIC EFFECT OF FACTOR Xa ON FIBROBLASTS

This study showed that fXa promotes fibroblast proliferation via a novel dual receptor system that involves binding of fXa to EPR-1 and activation of PAR-1 (chapter 4). This may have implications for our understanding of the mechanism of action of other proteases. Diagram 8 shows a putative model of interactions between fXa, EPR-1 and PAR-1.

6.2.1 PROTEASE RECEPTORS IN HUMAN FIBROBLASTS

A Dual Receptor System for Factor Xa

In chapter 4, we showed that fXa interacts with two cell surface receptors to stimulate fibroblast proliferation: EPR-1 and PAR-1.

EPR-1 seems to promote the proteolytic activity of fXa at the cell surface, whereas PAR-1 is the cell surface receptor that transduces the mitogenic signal of fXa across the membrane. Indeed, PAR-1 expression, activation and subsequent signalling events were necessary to transduce the mitogenic stimulus of fXa (sections 4.2.2 to 4.2.4; discussion in section 4.3.4). It is also known that PAR-1 mediates most of the effects of thrombin on fibroblast proliferation and procollagen production (Chambers *et al.* , 1998; Connolly *et al.* , 1996). Furthermore, the effects of fXa on fibroblasts are mediated primarily via activation of a Gαi protein-dependent pathway and cytosolic Ca2+ mobilisation similarly to thrombin. Thus fXa and thrombin utilise a common cell surface receptor, PAR-1, to transduce their effects on fibroblasts.

Other proteases have been shown to stimulate human fibroblast proliferation and their effects are mediated via cell surface receptors, but their mode of action is not always completely understood. It is possible that some of these proteases may use a similar receptor system similar to EPR-1/PAR-1 to transduce their effects. This will be discussed below.

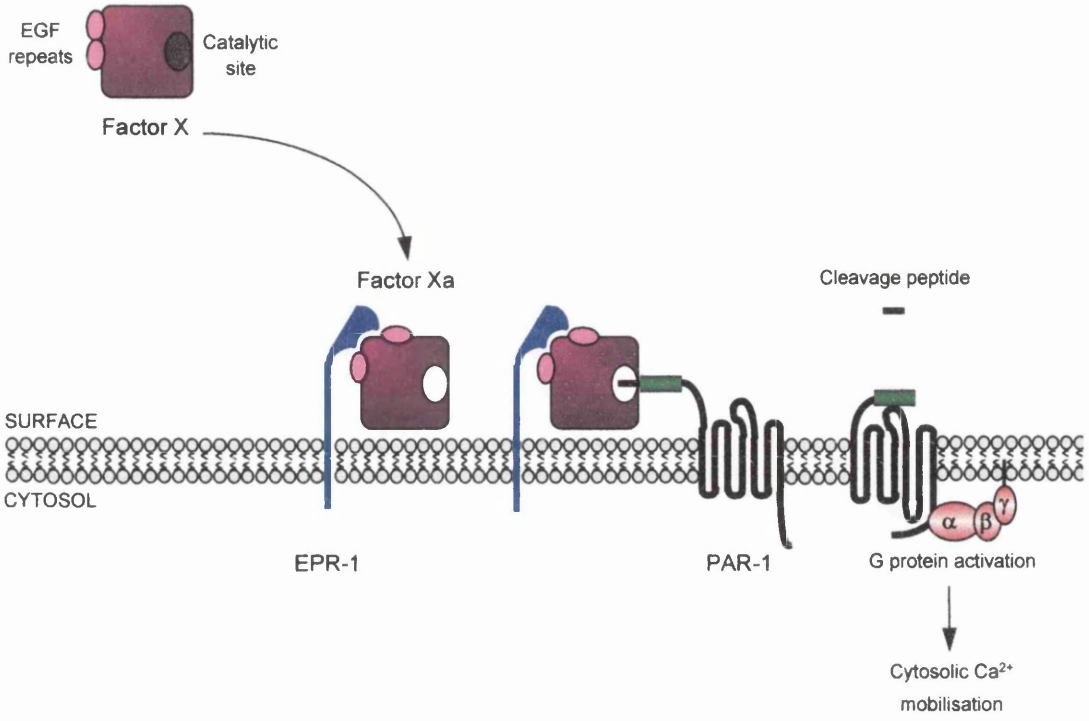


Diagram 8 **Factor Xa, EPR-1 and PAR-1: Putative interactions on the surface of human fibroblasts**

The data presented in this thesis suggests that four steps of protein interactions are necessary at the cell membrane for fXa to stimulate fibroblast proliferation. First, factor X requires to be activated by the factor VIIa/tissue factor complex (section 4.2.3.1). In this process, the protease acquires its' proteolytic activity and changes conformation, exposing an EPR-1-specific binding site otherwise hidden by two EGF repeats. Second, fXa docks onto EPR-1 on the cell surface (section 4.2.1). It is possible that EPR-1 also fulfils the function of cofactor and enhances the activity of fXa. Third, fXa proteolytically activates PAR-1 on the cell surface, in a similar fashion to thrombin (section 4.2.2). Following cleavage, the tethered ligand region of PAR-1 binds intramolecularly and stimulates a conformation change of the seven transmembrane domain receptor. Subsequently, the G protein subunits α, β and γ are recruited to the cell membrane and couple to PAR-1 (section 4.2.6). The phosphorylation events that ensue trigger second messenger pathways, rapid mobilisation of cytosolic free Ca^{2+} and eventually, cell proliferation (sections 4.2.3.2 and 4.2.5).

Urokinase-Type Plasminogen Activator Receptor

Urokinase-type plasminogen activator (uPA) is one of the principal serine protease that mediate fibrinolysis. uPA mediates fibroblast migration into FBN clots (Myohanen *et al.,* 1993). uPA is also known to stimulate cells via binding to its' cell surface receptor, urokinase-type plasminogen activator receptor (uPAR; Tkachuk *et al.,* 1996, for review). uPAR promotes cell surface plasminogen activation and ECM degradation (Roldan *et al.,* 1990). Occupancy of uPAR by uPA is localised at sites of contact between cells and the ECM (Myohanen *et al.,* 1993) and the receptor is thought to enhance uPA activity and plasminogen activation by promoting enzyme-substrate interactions at the cell surface (Ellis *et al.*, 1996). However, uPAR does not affect the catalytic activity of uPA per say.

By allowing cells to generate plasmin and remodel the ECM in their vicinity, cell surface-bound uPA enhances fibroblast migration and proliferation (Weaver *et al.*, 1997). Furthermore, uPA is thought to have direct cellular effects which includes the stimulation of fibroblast proliferation (De Petro *et al.,* 1994; Shetty *et al.,* 1996). This mitogenic effect is enhanced by binding of uPA to uPAR on fibroblasts (Anichini *et al.,* 1994; Shetty *et al.,* 1996) and its' proteolytic activity also contributes to this effect (Anichini *et al.,* 1994).

uPA does cleave the extracellular domain of uPAR and removes region necessary for uPA binding (Hoyer-Hansen *et al.,* 1992). However, this event does not promote the mitogenic effect of uPA. In contrast, cleavage of uPAR by uPA down-regulates its' binding to uPAR and provides a negative feedback mechanism. Thus the proteolytic activity of uPA must act on another substrate, possibly another cell surface receptor, is necessary to its' mitogenic effects on fibroblasts (Tkachuk *et al.,* 1996).

The complete mechanism of action of uPA has not been elucidated, but the findings of this thesis regarding the role of *accessory receptors* such as EPR-1 for fXa suggests that uPAR may fulfil a similar function for uPA. Moreover, it is possible that uPA activates a protease-activated receptor in with the contribution of uPAR as an accessory molecule. In addition, thrombin stimulates uPAR expression in vascular smooth muscle cells via activation of PAR-1 (Reuning and Bang 1992; Reuning *et al.,* 1994), suggesting a link between uPAR expression in smooth muscle cells and tissue repair in the vascular wall. Consistent with the hypothesis that uPAR is a mediator of connective tissue deposition, uPAR expression levels are also elevated in human fibroblasts from patients with fibrotic lung disease (Shetty *et al.,* 1996).

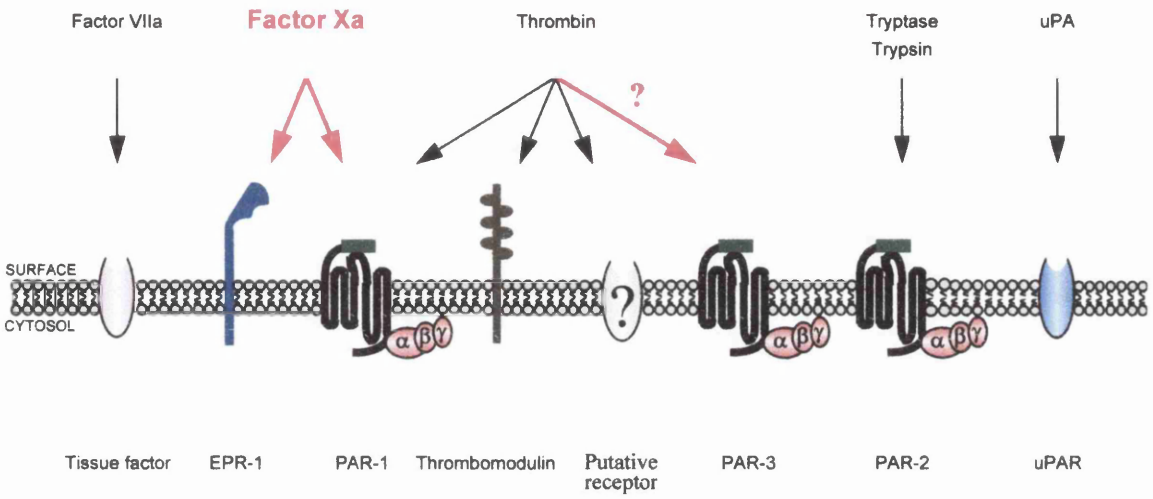


Diagram 9 Cell surface protease receptors in human fibroblasts

This diagram shows the protease receptors in human fibroblasts. The findings described in this thesis are illustrated in red.

- Tissue factor binds factor VIIa and promotes the assembly of the tenase complex. Ligation of tissue factor on fibroblasts leads to the production of growth factors with angiogenic properties. Second, PAR-1 is the high affinity surface receptor for thrombin in human fibroblasts. PAR-1 mediates the majority of the effects of thrombin on fibroblasts, including chemotaxis, mitogenesis and ECM production. Third, thrombomodulin is an alternative thrombin receptor on fibroblasts. Thrombomodulin modulates the substrate specificity of thrombin and transforms the physiological function of thrombin from procoagulant to anticoagulant. There is also some evidence that an additional thrombin receptor distinct from PAR-1 and thrombomodulin is present on fibroblasts. This putative receptor would mediate part of the mitogenic effects of thrombin. PAR-2 is the second protease-activated receptor present on fibroblasts and it mediates the proliferative effects of the inflammatory proteases tryptase and trypsin. Finally, uPAR is the receptor for the fibrinolytic protease uPA. uPAR enhances the mitogenic effects of uPA.
- Our data shows that EPR-1 is a fXa receptor present on fibroblasts. Furthermore, EPR-1 and PAR-1 form a dual receptor system that mediates the mitogenic effects of fXa in human foetal lung fibroblasts. Finally, this study also suggests that PAR-3 is present in human fibroblasts.

Alternative Thrombin Receptors

Results in chapter 4 and evidence in the literature suggest that PAR-1 is not the only cell surface receptor for thrombin in human fibroblasts. Thrombomodulin is an alternative thrombin receptor present in human fibroblasts (Shirayoshi *et al.,* 1993). As summarised in chapter 1, binding to thrombomodulin changes the substrate specificity of thrombin (Sadler, 1997). Thrombomodulin-bound thrombin preferentially activates the anticoagulant protein C and protein

S. On the other hand, thrombomodulin-bound thrombin becomes unable to cleave FGN or activate PAR-1 (Lafay *et al.,* 1998). This suggests that different protease receptors can play active roles in the regulation of protease activity, either reverse or promote the procoagulant and proliferative effects of thrombin and even transform the physiological role of a protease. Other receptors with similar effects on serine proteases may possibly exist in human fibroblasts.

Other PAR

This study showed by Northern blot analysis that PAR-3 mRNA is found in human foetal fibroblasts. The presence of an additional signalling receptor in human fibroblasts is also supported by the observation that thrombin stimulates cytosolic Ca^{2+} mobilisation in fXa-desensitised fibroblasts (section 4.2.3.3). This putative additional thrombin receptor in fibroblasts may be PAR-3, or a non-proteolytically activated receptor, as suggested by previous investigators (Carney *et al.,* 1992; Herbert *et al.,* 1994; Hollenberg *et al.,* 1996; Sower *et al.,* 1999). It is known that the proteolytic activation of PAR-1 (Garcia, 1992), PAR-2 (Nystedt *et al.,* 1995; Blackheart *et al.,* 1996), PAR-3 (Ishihara *et al.,* 1997) and PAR-4 (Xu *et al.,* 1998; Kahn *et al.,* 1999) are coupled to cytosolic Ca^{2+} mobilisation. Taken together, these data suggest that both PAR-1 and PAR-3 are present and activated by thrombin in human fibroblasts, and lead to cytosolic Ca^{2+} mobilisation. Finally, PAR-2 was also detected in our human fibroblasts. This receptor may mediate the mitogenic effects of tryptase and trypsin-related serine proteases as suggested previously in fibroblasts (Ruoss *et al.,* 1991; Molino *et al.,* 1997b) and smooth muscle cells (Bono *et al.,* 1997b).

Other Coagulation Cascade Protease Receptors

Tissue factor is the cell surface receptor for factor VIIa on fibroblasts (Bloem *et al.,* 1989). Tissue factor mediates the assembly of the tenase complex on the surface of fibroblasts and triggers the extrinsic pathway of the coagulation cascade (section 1.1.2.1). Factor VIIa stimulates the expression of vascular endothelial growth factor by human fibroblast and play a role in endothelial cells growth and angiogenesis through this mechanism (Ollivier *et al.,* 1998). There is also some evidence that the ligation of tissue factor with factor VIIa may be linked to the stimulation of poly(A) polymerase mRNA levels in human fibroblasts (Pendurthi *et al.,* 1997) but little is known on the physiological significance of this effect. However, it is interesting to note that the stimulation of vascular endothelial growth factor on fibroblasts is dependent upon the proteolytic activity of factor VIIa. Although the precise function for the catalytic site of factor VIIa has not been found, one could

speculate that the mechanism of action of factor VIIa might be similar to that of fXa and involve the activation of a protease-activated receptor as well as binding to tissue factor.

Factor XIIa is another serine protease of the coagulation cascade that initiates the intrinsic pathway of the coagulation cascade. Factor XIIa is mitogenic for liver cells (Scmeilder-Shapiro*et al.*, 1991). Both factor XII and proteolytically active factor XIIa are mitogenic to Hep2 cells and their effects appear to be mediated via the EGF repeats present in their sequence, not via the catalytic site of the protease (Gordon *et al.*, 1996). It has been suggested that factor XIIa may share a cell surface receptor with high molecular weight kininogen in endothelial cells (Reddigari *et al.*, 1993), but this putative receptor has not been identified.

The anticoagulant serine protease activated protein C possesses a cell surface receptor in endothelial cells (Fukudome *et al.*, 1996). Mononuclear phagocytes also possess a cell surface receptor for activated protein C that is distinct from that of endothelial cells (Hancock *et al.*, 1996). A similar receptor also appears to mediate the inhibitory effect of activated protein C on phagocyte activation (Hancock *et al.*, 1996) and may help localise activated protein C to the vascular endothelium (Bangalore *et al.*, 1994). These receptors have not been fully characterised.

Summary

This thesis showed that the mitogenic effects of fXa on fibroblasts are mediated by EPR-1 and PAR-1. Since both receptors appear to mediate the effect of fXa, they may form a novel dual receptor system comprised of an accessory receptor and a signalling protease-activated receptor. It is possible that other serine proteases with mitogenic effects on fibroblasts and similarities with fXa such as factor VIIa, activated protein C or uPA might work through a similar receptor system involving a PAR.

6.2.2 Role of PAR-1 and EPR-1 in Tissue Repair and Fibrosis

Evidence in Animal Models

The observation that fXa and thrombin both activate PAR-1 raises the question of the importance of this receptor in tissue repair and fibrotic diseases.

PAR-1-deficient mice showed near-normal tissue repair in incisional models of wound healing, including normal healing rate, normal tensile strength and normal re-epithelialisation (Darrow *et al.*, 1996; Connolly *et al.*, 1997). If PAR-1 contributes to tissue repair, this implies that other mechanisms take over its' function in genetically modified animals. However, complete

PAR-1 deficiency results in a mortality rate of 87% (Connolly *et al.*, 1996) to 93% (Darrow *et al.*, 1996) *in utero*, suggesting that PAR-1 plays a role during embryogenesis. The 7% to 13% of animals that survive early development may have uncommon genotypes that allow them to compensate for PAR-1 deficiency and reach adulthood. In consequence, it is possible that the animals that are available for biomedical research have been naturally selected for their ability to withstand PAR-1-deficiency and develop other means of response against injury. In these conditions, it may not be entirely surprising that these mice show little phenotypic differences in experimental models of tissue repair (Darrow *et al.*, 1996; Connolly *et al.*, 1997). However, it is possible that there may be redundant repair mechanisms and protease receptors.

In addition, different PAR can mediate a single physiological function as in the case of PAR-1 and PAR-4 activation and human platelet activation by thrombin (Kahn *et al.*, 1999). Other protease receptors than PAR-1 may mediate the effects of coagulation cascade proteases in mesenchymal cells and compensate for the absence of PAR-1. However, our data obtained in desensitised human fibroblasts and PAR-1-deficient mouse fibroblasts suggest that PAR-1 is the main signalling cell surface receptor for thrombin (Connolly *et al.*, 1996) and fXa.

PAR-1 and EPR-1 expression levels are elevated in smooth muscle cells following arterial injury (Takada *et al.*, 1995; Herbert *et al.*, 1998). Moreover, the inhibition of EPR-1 with specific blocking antibodies abrogated smooth muscle cell proliferation and neo-intimal proliferation by about 70% in a model of arterial injury in the rabbit (Herbert *et al.*, 1998). Targeting PAR-1 with blocking antibodies in similar models also reduced smooth muscle cell proliferation and neo-intimal formation by about 50% (Takada *et al.*, 1995). There are no reports investigating the expression and role of PAR-1 or EPR-1 in animal models of tissue fibrosis. However, the evidence obtained for vascular repair *in vivo* supports the idea that PAR-1 and EPR-1 may play a role during mesenchymal proliferation and ECM deposition in normal tissue repair and fibrosis.

Clinical Evidence

PAR-1 mediates the effects of thrombin on proliferation and procollagen production by fibroblasts (Trejo *et al.*, 1996; Chambers *et al.*, 1998) and smooth muscle cells *in vitro* (Herbert *et al.*, 1992; Dabbagh *et al.*, 1998). In addition, PAR-1 levels are elevated in several types of fibrosis of the vessel wall, including atherosclerotic vascular lesions (Nelken *et al.*, 1992) and restenosis due to balloon angioplasty as well as vascular injury (Wilcox *et al.*, 1994). The up-regulation of PAR-1 in mesenchymal cells following vascular injury occurs rapidly and persists throughout neo-intimal formation (Wilcox *et al.*, 1994; Maruyama *et al.*, 1997; Baykal *et al.*, 1995, for review).

PAR-1 expression has also been associated with synovial cell proliferation in patients with rheumatoid arthritis associated with excessive fibroblast proliferation and ECM deposition (Shin*et al.,* 1995; Morris *et al.*, 1996). Elevated PAR-1 expression is also characteristic of severe forms of glomerulonephritis with FBN deposition (Sraer and Rondeau, 1996; Rondeau *et al.,* 1996, for review) and chronic liver injury (Marra *et al.,* 1998). This suggests that PAR-1 may also play a role in fibroproliferative disorders of the kidney and liver. Collectively, these clinical observations suggest that PAR-1 expression is associated with connective tissue formation.

Summary

PAR-1 and EPR-1 expression have been associated with tissue fibrosis in the vascular wall in humans. However, genetically modified animals have provided little clues regarding the role of these receptors in tissue repair and fibrosis and it remains to be determined whether PAR-1 and EPR-1 contribute to human normal tissue repair processes and disease.

6.3 THE LIMITATIONS OF THIS THESIS

This thesis identifies for the first time several aspects of the role of coagulation cascade products and their mode of action in fibroblasts. However, this vast subject has only been partially covered and some of the major points that have not been investigated will be discussed.

6.3.1 OTHER COAGULATION CASCADE PEPTIDES

This study investigated the effects of several coagulation cascade products on fibroblast proliferation. Since the coagulation factors of the extrinsic pathway are activated following tissue injury and during tissue fibrosis, they have been selected for this work. However, there are other coagulation factors that have not been investigated, including factor XIa, XIIa, peptides of the kininogen-kallikrein system and the anticoagulant factors activated protein C and activated protein S (section 1.2). All these peptides are activated early in the intrinsic pathway, but their activation mechanism is less well understood than that of factors VIIa, Xa and thrombin. The levels of activation of these factors in tissues during normal tissue repair or in disease settings are not known. Their plasma levels are sometimes measured as an indicator of blood coagulation. However, it would be interesting to complete the present work to study their effects on fibroblast

proliferation. Indeed, two studies suggested that factor XIIa and protein S stimulate proliferation in HepG2 and smooth muscle cells via their EGF-like repeats (Schmeidler-Sapiro *et al.*, 1991; Gasic *et al.*, 1992).

6.3.2 Other Growth Factors and Cytokines Stimulated

This thesis demonstrates that PDGF production and autocrine stimulation is necessary to the mitogenic effects of fXa on fibroblasts. However, there are numerous other cytokines and growth factors that are mitogenic to fibroblasts. Some of them, such as bFGF, TNF-a, IL-1, IL-6, IL-8 are stimulated by thrombin and fXa in fibroblasts and other cells (sections 3.1.1.3 and 3.1.2.1). These peptides may have effects on fibroblasts that are still unknown, or possibly contribute to proliferation via novel mechanisms. Thus, it can not be assumed from the present study that PDGF is the principal cytokine stimulated by fXa in fibroblasts, nor that proliferation represents the prevalent effects of coagulation cascade products on these cells. Indeed, the actual levels of PDGF produced by fibroblasts stimulated by fXa have not been determined.

In addition, fXa may have other effects than simply stimulating the expression of PDGF in fibroblasts. FXa may have more complex effects on growth factor and cytokine activation, release from extracellular stores segregated around the ECM, or cell surface receptor expression, all of which may affect fibroblast proliferation. These aspects have not been investigated.

6.3.3 Other Aspects of Receptor Activation

It has been shown in chapter four that fXa binds to EPR-1 and activates PAR-1. Furthermore, binding to EPR-1 is not sufficient to stimulate fibroblast Ca^{2+} mobilisation and proliferation. However, several aspects have not been fully examined.

First, it is possible that binding of fXa to EPR-1 may itself have signalling effects and cellular consequences that are not linked to Ca^{2+} mobilisation and proliferation in fibroblasts.

Second, this study did not establish that fXa activates PAR-1 through proteolytic cleavage in a similar manner to thrombin. Demonstrating that fXa cleaves PAR-1 at the expected activation site would be a convincing piece of information to confirm that fXa activates the receptor in fibroblasts. I attempted to demonstrate cleavage of PAR-1 after treatment of fibroblasts with fXa by immunofluorescence, confocal laser-scanning immunomicroscopy and fluorescence automatic cell sorting (FACS) using specific antibodies directed against the cleavage site and the tethered

ligand of PAR-1 provided by Dr L. Brass, University of Pennsylvania, Philadelphia, PA, USA; Hoxie *et al.,* 1993; Brass *et al.,* 1994; Ramachadran *et al.,* 1997). However, human foetal lung fibroblasts proved difficult to handle and an ill-suited cell type for such studies due to their very thin and flat morphology and granulous appearance in suspension. Different fixation methods with either paraformaldehyde or methanol:acetone mixes did not alleviate theese difficulties and the experiments remained inconclusive. Alternatively, the cleavage product generated upon cleavage of PAR-1 by fXa should be measured in the supernatant of treated cells. This may be possible using enzyme-linked immunosorbant assays (ELISA) based on specific anti-PAR-1 tethered ligand antibodies (Molino *et al.,* 1997a; Ramachadran *et al.,* 1997).

Third, the localisation of EPR-1 and PAR-1 has not been fully examined so that it remains unknown whether the two receptors come together or cluster to transduce the mitogenic effect of fXa in fibroblasts. Immunofluorescence analysis of the cells before and at the time of stimulation with fXa would be useful to address this question. This would also complement the Northern blot and Western blot studies performed in this thesis and help determine the presence of the receptors at the cell membrane. Immunofluorescence or FACS would also allow the observation of their movements between intracellular stores and the cell surface.

Fourth, immunofluorescence would give a gross indication of the abundance of EPR-1 in human fibroblasts, but a Scatchard plot analysis of ligand binding analysis with radiolabelled versus unlabelled fXa would be necessary to determine the abundancy of EPR-1 in fibroblasts.

Finally, the signalling events that link activation of PAR-1 by fXa to Ca2+ mobilisation and proliferation have not been researched. This would provide some precious information about the signalling events linked to fXa and allow the comparison with PAR-1 activation by thrombin. For instance, it is known that protease-activated receptors are linked to different G proteins, including Gi, Gq and Go proteins (section 4.1.1.3). It is possible that fXa and thrombin do not cause the association of PAR-1 with the same G proteins, suggesting that the two proteases may have specific effects although they activate the same protease receptor in human fetal lung fibroblasts.

6.3.4 *IN VIVO* RELEVANCE

The work presented here has been performed entirely *in vitro.* Therefore, it remains unknown whether the observations made are relevant to phyiological settings. This question of physiological relevance can only be addressed by experiments performed *in vivo* so that all the

physiological factors, known and unknown, that contribute to tissue repair, fibrosis and fibroblast behaviour are found in a single environment where they attain a natural, integrative and dynamic equilibrium.

For instance, the comparative relevance of the growth factors stimulated by fXa and thrombin in fibroblasts (such as PDGF) versus those secreted by other cells in their vicinity has not been investigated. The actual relevance of these stimulations to fibroblast proliferation in physiological conditions could only been determined by *in vivo* experiments. Experiments will be suggested in the following sections.

6.4 FUTURE STUDIES

The novel findings of this thesis should be pursued in several ways: First, the effects and the mode of action of coagulation cascade proteases on fibroblasts should be further described. Second, the experiments performed for this thesis were carried out *in vitro* and questions are raised regarding the relevance of these effects *in vivo*. These issues will be approached in two sections: One devoted to coagulation cascade proteases and one to their receptors.

6.4.1 COAGULATION CASCADE PROTEASES IN TISSUE REPAIR

Since thrombin stimulates fibroblast proliferation and procollagen production *in vitro* and *in vivo*, it would be interesting to investigate the profibrotic effects of fXa in animal models.

First, this could be done by measuring skin fibroblast proliferation and collagen deposition *in situ* after intradermal injection of fXa. Alternatively, the effect of specific fXa inhibitors on fibroblast proliferation and collagen deposition could be assessed in experimental models of connective tissue formation. The most informative experiments could be performed in tissues containing an extensive vascular bed, such as the lungs, where tissue injury results in prominent activation of the coagulation cascade. The model of bleomycin-induced lung fibrosis would be ideal for such studies (Shen *et al.*, 1988). The effects of fXa inhibitors on connective tissue formation could also be investigated in the skin using the incisional (Pierce *et al.*, 1989) and full thickness excisional wound healing models (Pierce *et al.*, 1991b). Subcutaneous synthetic sponge implant models of granulation tissue formation could also be employed to measure connective tissue deposition after injection of fXa (Lepisto *et al.*, 1996). Such studies would provide valuable insights on the importance of fXa and coagulation cascade proteases during connective tissue formation in wound healing and tissue fibrosis.

Measuring FBN deposition and blood parameters, as well as fibroblast proliferation and matrix deposition may help distinguish between the pro-coagulant and the direct cellular effects of coagulation cascade factors. Inhibiting the early steps of the coagulation cascade, such as factor VIIa or Xa, would be a particularly good strategy. Indeed, inhibiting fXa *in vivo* is an effective antithrombotic strategy that does not cause the bleeding complications inherent to thrombin inhibition (Wong *et al.,* 1996; Schwartz *et al.,* 1996). Furthermore, targeting fXa would provide the double advantage of repressing its cellular effects as well as FBN deposition and thrombin formation, thereby inhibiting both direct and indirect effects of the coagulation cascade. This could provide a basis for the development of novel therapeutic strategies for fibrotic diseases associated with extensive and persistent activation of the coagulation cascade.

6.4.2 Coagulation Cascade Protease Receptors in Tissue Repair

This thesis shows that PAR-1 and EPR-1 mediate the effects of fXa in human fibroblasts. It would be interesting to gather more information about the role and mechanism of action of this dual receptor system *in vitro* and *in vivo*.

In Vivo Studies

First, PAR-1 mediates the mitogenic effects of both thrombin and fXa on fibroblasts (chapter 4) and these proteases are the most potent fibroblast mitogens activated during blood coagulation (chapters 3 and 5). Thus PAR-1 may mediate a significant part of the mitogenic effects of the coagulation cascade on fibroblasts. To evaluate the role of PAR-1 in connective tissue deposition *in vivo* and complement the findings of this thesis, EPR-1 and PAR-1 could be targeted with specific neutralising antibodies, short peptides or small molecular weight compounds in models of tissue repair and fibrosis. Since specific PAR-1 blocking antibodies down-regulate neo-intimal formation in models of arterial injury (Takada *et al.,* 1997), the same antibodies could be evaluated in bleomycin-induced pulmonary fibrosis (Shen *et al.,* 1988 and section 6.1.3).

Alternatively, one could measure tissue fibrosis after topical delivery of EPR-1 and PAR-1 agonist peptides to organs such as the lungs. These peptides could mimic regions of fXa which are known to bind specifically to EPR-1 (Cirino *et al.*, 1997; Herbert *et al.,* 1998) or PAR-1-activating peptides (Hollenberg *et al.*, 1996; Chambers *et al.,* 1998). This would provide information about the role of these receptors *in vivo*.

In Vitro Studies

Another possible direction of research to add to the findings of this thesis is to characterise further the interaction between fXa and the cell surface receptors EPR-1 and PAR-1. The regions of fXa that interact with PAR-1 to allow cleavage of the receptor should be further investigated. To cleave PAR-1, thrombin utilises a binding site within PAR-1with high sequence similarity with hirudin (Blackhart *et al.*, 1994). Binding to the hirudin-like region of PAR-1 produces a change of conformation within the catalytic site of thrombin. This allows the amino terminus of PAR-1 to fit into the catalytic groove of the protease and activation of the receptor (Ishii *et al.*, 1995). In contrast, hirudin does not interfere with the mitogenic effects of fXa (section 3.2.2.2).

The interaction between fXa and PAR-1 needs to be further investigated. This could be done by confirming the colocalisation of PAR-1 and EPR-1 after exposure of fibroblasts to fXa. This could be established by confocal microscopy using specific monoclonal antibodies. Neutralising antibodies directed against various regions of the protease and the receptor could also be employed to abrogate this interaction and identify the regions involved in this interaction.

Other questions that need to be researched are whether fXa activates PAR-1 by proteolytic activation and whether it utilises the same cleavage site as thrombin in PAR-1. This could be done by measuring levels of PAR-1 cleavage product in the supernatant of fibroblasts treated with fXa *in vivo* using enzyme-linked immunosorbant assays or by HPLC separation of the short peptide. Sequencing the PAR-1 cleavage product would also be informative.

Summary

The findings of this thesis could be pursued in several ways. First their relevance to tissue repair events should be investigated *in vivo*. Second, the mode of interaction and activation of PAR-1 by fXa should be further studied. Successful results in these areas could provide a strong basis for the development of possible novel drugs that enhance tissue repair or alleviate tissue fibrosis.

6.5 POSSIBLE MEDICAL APPLICATIONS FOR ANTITHROMBOTICS

The inhibition of the coagulation cascade is a classical approach to prevent and treat thrombotic conditions known as *anticoagulant therapy*. However, the recent discovery of the

cellular effects of coagulation cascade factors may reveal new therapeutic purposes for antithrombotics.

6.5.1 Coagulation Cascade Protease Inhibitors

Classical Coagulation Cascade Inhibitors

Anticoagulant therapy is used in patients to treat or prevent the development of thromboembolic disease. It can be prescribed prophylactically for major surgery to prevent venous thromboembolism. It is also used in the treatment of deep-vein thrombosis, pulmonary embolism and atrial fibrillation. It is also useful during and after treatment by percutaneous coronary angioplasty, aiming to prevent subsequent arterial thrombus formation and re-occlusion (Beijering *et al.,* 1996, for review).

The main anticoagulant strategies involve the administration of *warfarin-* or *heparin-*related compounds. These drugs aim to inactivate vitamin K-dependent coagulation cascade proteases to interrupt thrombin generation (for review, see Harker *et al.,* 1997). Warfarin blocks the post-translational modification of Glu into Gla residues necessary for the activity of factor VII, IX, X and prothrombin (Pineo and Hull, 1993 for review and section 1.1.2.2 for details on Gla residues). Warfarin is used as a prophylactic in chronic conditions for its' long term and profound effects on multiple coagulation factors. However, warfarin can cause severe drug reactions.

Heparin acts as a cofactor for endogenous antithrombin III and catalyses the inhibition of thrombin and fXa. Heparin is used in acute situations to obtain rapid anticoagulation. However, standard heparin has proven to be only moderately effective, poorly bioavailable and its' effects are variable and unpredictable (Harenberg *et al.,* 1997).

Novel Coagulation Cascade Inhibitors

Novel antithrombotics are designed to be more specific and have fewer side effects than warfarin and heparin. They aim principally to inhibit specific coagulation factors that play a pivotal function in the cascade, such as fXa and thrombin. Since the activation of fXa is critical to thrombin generation, it constitutes an advantageous target for antifibrotic as well as antithrombotic therapy.

A number of small molecular weight inhibitors of fXa and thrombin have been discovered recently by structure-function relationship analysis, leading to the discovery of fractionated heparin (Hemker *et al.*, 1996, for review) and synthetic oligosaccharide heparin mimetics (Petitou

et al., 1999) which are more specific for fXa than standard heparin. A number of novel fXa and thrombin inhibitors have also been produced by industrials through peptidomimickry of natural inhibitors purified from haematophagous animals such as *tick anticoagulant peptide* (Jordan *et al.*, 1990; Jordan *et al.*, 1992), leech *hirudin* (Fenton *et al.*, 1998, for review) and *antistasin* (Tuszynski *et al.*, 1987) and *nematode anticoagulant peptide* (Cappello *et al.*, 1993).

The direct inhibition of thrombin's proteolytic activity has proved successful to limit re-occlusion after acute myocardial infarction and thrombolysis therapy (Zeymer *et al.*, 1996). However, it is associated with a high risk of bleeding complications. The inhibition of fXa is at least as effective as that of thrombin at doses of inhibitor which only prolong clotting time moderately (Wong *et al.*, 1996). In addition, the transient inhibition of fXa versus thrombin presents the advantage of long term antithrombotic protection and attenuates markedly whole clot procoagulant activity (Parger *et al.*, 1995; McKenzie *et al.*, 1996; Kotze *et al.*, 1997).

The efficacy and safer clinical profile of specific fXa inhibitors have been observed in experimental models of reperfusion after arterial injury in the dog (Benedict*et al.*, 1993; Lynch *et al.*, 1994) and deep vein thrombosis in the rabbit (Hollenbach *et al.*, 1994). Specific inhibitors of fXa are considerably safer to prescribe for their pharmacokinetics are better understood and their effects more predictable (Petitou *et al.*, 1999). In summary, fXa is an advantageous target to inhibit the effects of the coagulation cascade.

6.5.2 Novel Applications for Coagulation Cascade Protease Inhibitors

Several natural inhibitors including tick anticoagulant peptide and antistasin have been conclusively used in this thesis to demonstrate the contribution of the catalytic site of fXa in its' mitogenic effects on fibroblasts *in vitro*. This suggests that fXa inhibitors and possibly a range of antithrombotics could be used to treat tissue fibrosis. However, the possibility that fXa inhibitors may be useful to limit excessive fibroblast proliferation and treat tissue fibrosis has not been directly investigated.

There is comparatively more evidence that excessive activation of fXa and thrombin in the coagulation cascade is associated with smooth muscle cell proliferation and ECM production in the vessel wall following vascular injury (Ghigliotti *et al.*, 1998). Thus the majority of research on potential novel applications for anticoagulants focused on vascular disorders. For instance, local adenoviral delivery of hirudin limited neo-intimal formation after arterial injury (Rade *et al.*, 1996). Similarly, the administration of heparin or small molecular weight fXa inhibitors abolished

FBN deposition (Heras *et al.,* 1992), smooth muscle cell proliferation and matrix production (Chajara *et al.,* 1994) in the vessel wall in models of arterial injury. The specific inhibition of fXa has also proven beneficial over antithrombin therapy to alleviate common complications linked to open heart surgery (Laporte *et al.*, 1999) and to promote long term patency of vascular prosthetic while minimising the risk of haemorrhage (Oltrona *et al.,* 1996; Ritter *et al.,* 1998). Taken together, these data demonstrate that fXa inhibitors can be used successfully in patients.

6.5.3 PROTEASE RECEPTOR INHIBITORS

Several studies aimed to neutralise PAR-1 in animal models of smooth muscle cell hyperplasia after arterial injury and there is some discordance regarding the efficacy of this approach. One group reports inhibiting smooth muscle cell proliferation *in vitro* with antisense oligodeoxynucleotides directed against PAR-1 mRNA (Chaikof *et al.,* 1995). Another group reports having no success with this method (Herbert *et al.,* 1997). In contrast, one group claimed to obtain about 50% inhibition of neo-intimal formation and 50% down-regulation of smooth muscle cell proliferation by injecting blocking anti-PAR-1 antibodies in the area of vascular injury (Takada *et al.,* 1997). The inhibition of PAR-1 *in vivo* with specific antibodies against the exosite of the receptor also gave promising results in a model of arterial thrombosis in monkeys (Cook *et al.,* 1995).

EPR-1 Expression has also been associated with smooth muscle cell hyperplasia and neo-intimal formation after arterial injury (Herbert *et al.,* 1998), suggesting that the mitogenic effect of fXa on smooth muscle cells contributes the progression of vascular diseases. Neo-intimal formation in models of arterial injury was reduced by about 70% by using blocking EPR-1 antibodies *in vivo* (Herbert *et al.,* 1998) supporting the idea that EPR-1 mediates the effects of fXa in mesenchymal cells.

Taken together, these observations suggest that PAR-1 and EPR-1 mediate the effects of fXa in mesenchymal cells *in vivo.* Targeting these receptors may be a therapeutic option for diseases characterised by excessive mesenchymal proliferation and connective tissue deposition.

CHAPTER 7

REFERENCES

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