THE REGULATION OF PROTEASES AND
MECHANICAL LOADING DURING FIBROBLAST
POPULATED COLLAGEN LATTICE CONTRACTION.

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ABSTRACT

Dermal wound healing is a complex multi-stage process involving many cell types, several cell-matrix interactions, and is influenced by internal and external environmental factors. Of these, mechanical stimulation of the wound region has been shown to be highly significant influencing tissue formation and remodelling.

The hypothesis here has been “Protease production is closely associated with external mechanical stimulation of fibroblast populated collagen lattices”. This was investigated using three *in vitro* lattice models of mechanical loading (1) non-load, (2) endogenous static load, and (3) exogenous loading (cyclical and continuous loads). Exogenous loading regimes were applied across the lattice using a computer controlled drive unit incorporated into a culture system known as tension-Culture Force Monitor. Proteases released into the media were analysed by zymography and Western blotting. Whole lattices were examined by immunohistochemistry.

A wide range of Matrix metalloproteases (MMPs) and Serine proteases were detected in media from human dermal and pathological (scar and keloid) fibroblast cultures following mechanical loading. Different profiles were present for each of the enzymes monitored. Substantial increases of MMP-1 (collagenase) and MMP-9 (gelatinase B), and tissue-type plasminogen activator (tPA) were demonstrated following exogenous loading, whilst, MMP-2 (gelatinase A) was mostly unaffected. MMP-3 (stromelysin) and urokinase-type plasminogen activator (uPA) were mainly reduced following loading regimes.

In conclusion, fibroblasts within collagen matrices produced different protease responses following mechanical loading, indicating that each protease had a differential role relevant to cell-matrix interactions—attachment, locomotion, and contraction. Clinically, scar contraction following injury or surgery could be reduced by protease production influencing matrix remodelling.
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<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>APMA</td>
<td>aminophenylmercuric acetate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CFM</td>
<td>Culture Force Monitor</td>
</tr>
<tr>
<td>COL</td>
<td>cyclical over load</td>
</tr>
<tr>
<td>CUL</td>
<td>cyclical under load</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified Eagles medium</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetra-acetic acid</td>
</tr>
<tr>
<td>FCS</td>
<td>fetal calf serum</td>
</tr>
<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FPCL</td>
<td>fibroblast populated collagen lattice</td>
</tr>
<tr>
<td>HAR</td>
<td>high aspect ratio</td>
</tr>
<tr>
<td>LAR</td>
<td>low aspect ratio</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteases</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane type matrix metalloproteinase</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoreses</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methyl sulphonyl fluoride</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>t-CFM</td>
<td>tension-Culture Force Monitor</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinases</td>
</tr>
<tr>
<td>TPA</td>
<td>12-O-tetradecanoylphorbol-13-acetate</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue type plasminogen activator</td>
</tr>
<tr>
<td>uPA</td>
<td>urokinase type plasminogen activator</td>
</tr>
<tr>
<td>uPAR</td>
<td>urokinase type plasminogen activator receptor</td>
</tr>
<tr>
<td>3-D</td>
<td>three dimensional</td>
</tr>
</tbody>
</table>
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CHAPTER 1- INTRODUCTION

1.1 - WOUND HEALING - AN OVERVIEW

Normal wound healing is a complex, dynamic, and interactive process with cellular and extracellular matrix (ECM) components responding to tissue injury in an intricate and organised manner. Healing is initiated at the precise moment tissue trauma occurs to optimise wound closure, providing a barrier against bacterial infection, and continues until normal tissue structure is restored or a scar is formed. This process can be divided into three arbitrary, overlapping phases: the inflammatory, proliferative and remodelling phase. The movement of cells into the wound occurs in a chronological order that corresponds to the functions required at the different phases of healing (Tahery and Lee, 1989; Mast, 1992; Clark, 1996).

Wound healing and scar formation are basic properties of all multicellular organisms and are required for survival (Tahery and Lee, 1989). However, alterations in the normal repair process can lead to undesirable outcomes, prolongation of the healing response or total failure to heal, such as strictures, fibrosis, and ulcers (Clark, 1996; Witte and Barbul, 1997). Here, the goal of wound healing research is for advancement in wound care management leading to accelerated rates of normal wound repair following injury and surgery, eventually substituting tissue regeneration for scar formation (Clark, 1996).
Normal wound healing occurs throughout the body. This overview, however, has been restricted mainly to dermal healing and is focused mostly on fibroblasts.

1.1.1. Normal skin composition

Normal skin is composed of a keratinised, stratified, squamous epithelium, and an underlying connective tissue that constitutes the epidermis and dermis, providing support and nourishment (McCarthy et al., 1996; Martin, 1997). The epithelium is separated from the underlying tissue by a basal lamina and reticular laminar, representing the dermal-epidermal junction. The dermis constitutes dense irregular connective tissue, and is enriched in types I and III collagen, dermatan, chondroitin, heparan sulphate, and keratin sulphate proteoglycans (Pringle et al., 1985). The proteoglycan core proteins present within dermis include versican, decorin, and biglycan (Lennon et al., 1991; Willen et al., 1991; Yeo et al., 1991; Schonherr et al. 1993; Scholzen et al., 1994; Zimmermann et al., 1994). Fibronectin in the dermis is associated with either collagen fibres or it exists as independent fibrils within the ground substance (McCarthy et al., 1996).

1.2 - INFLAMMATION PROCESS

1.2.1. Platelets

Upon injury the composition of the ECM changes dramatically (Grinnell et al., 1981; Repesh et al., 1982; Gailit and Clark, 1994). The epidermal and dermal elements are disrupted and the cutaneous vasculature is severed causing peripheral blood cells to
spill into the wound site (Mast, 1992). Preceding the inflammatory phase is hemostasis. Platelets are the first cellular elements to enter the wound site. In addition to their hemostatic role, they influence the subsequent cellular response within the wound, by secreting multiple mediators, including growth factors.

Growth factors are polypeptide molecules that during wound healing are involved with controlling growth, differentiation, metabolism of cells, stimulate cellular proliferation, chemotaxis, haptotaxis, angiogenesis, protein expression, and enzyme production during each of the three phases (McGrath, 1990; Rothe and Falanga, 1992; Steed, 1997). They are present in minute concentrations, yet exert a powerful local influence on wound repair, interacting with specific cell surface receptors, leading to distinct responses determined by the receptor mediated signal transduction pathways within the target cells.

As platelets come into contact with damaged collagen and other tissue debris, aggregation, degranulation, and activation of the coagulation and complement pathways occur. The contact between collagen and platelets, as well as the presence of thrombin, fibronectin, and their fragments, results in the release of cytokines from platelet α-granules such as platelet activating factor, fibronectin, serotonin (Mast, 1992; Wahl and Wahl, 1992; Witte and Barbul, 1997), platelet-derived growth factor (PDGF) a chemoattractant for smooth muscle cells (Grotendorst et al., 1983) and
fibroblasts (Seppa et al., 1982), and transforming growth factor-β (TGF-β) a chemoattractant for inflammatory cells and fibroblasts (Table 1.2.1.).

The locally formed fibrin clot serves as scaffolding for invading cells, such as neutrophils, monocytes, fibroblasts, and endothelial cells, to attach and migrate into the wound site (Schilling, 1976; Kurkinen et al., 1980). Specifically, fibrin in conjunction with fibronectin act as a provisional matrix for the influx of monocytes (Ciano et al., 1986; Lanir et al., 1988) and fibroblasts (Grinnell et al., 1980; Knox et al., 1986; Brown et al., 1993).
TABLE 1.2.1.- Growth Factors in Wound Repair.

(Steed, 1997).

<table>
<thead>
<tr>
<th>GROWTH FACTOR</th>
<th>CELL SOURCE</th>
<th>ACTIVITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transforming growth factor-α (TGF-α)</td>
<td>Platelets, macrophages, keratinocytes</td>
<td>Activates neutrophils, fibroblast mitogen, stimulates angiogenesis</td>
</tr>
<tr>
<td>Transforming growth factor-β (TGF-β)</td>
<td>Platelets, macrophages, lymphocytes</td>
<td>Stimulates angiogenesis and fibroplasia, induces proliferation of many different cells</td>
</tr>
<tr>
<td>Platelet derived growth factors (PDGF)</td>
<td>Platelets, macrophages, keratinocytes, endothelial cells</td>
<td>Chemoattractant for neutrophils, fibroblasts, mitogen for smooth muscle cells and fibroblasts</td>
</tr>
<tr>
<td>Fibroblast growth factor (FGF)</td>
<td>Macrophages, neural tissue, nearly ubiquitous</td>
<td>Stimulates endothelial cell growth, mitogen for mesodermal and neuroectodermal derived cells</td>
</tr>
<tr>
<td>Epidermal growth factor (EGF)</td>
<td>Platelets, keratinocytes, salivary gland</td>
<td>Mitogen for keratinocytes, endothelial cells, and fibroblasts</td>
</tr>
<tr>
<td>Insulin like growth factor (IGF)</td>
<td>Liver</td>
<td>Mitogen for fibroblasts, stimulates smooth muscle cells, lymphocytes, chondrocytes</td>
</tr>
</tbody>
</table>
1.2.2.- Neutrophils

Following clot formation, secondary vasodilatation and increased capillary permeability develops, resulting in the initiation of acute inflammation as neutrophils enter the wound site.

Neutrophils are the first wave of migrating cells to enter the wound. Increased permeability due to inflammation and release of prostaglandins together with a concentration gradient of chemotactic factors, interleukin-1, tumour necrosis factor-α, fibrinopeptides cleaved from fibrinogen by thrombin; fibrin degradation products; complement factors- C5a arising from activated classical or alternative complement cascades; leukotriene B₄ released by activated neutrophils; platelet activating factor released from endothelial cells or activated neutrophils; PDGF and platelet factor IV released from platelets, and bacterial products- formyl methionyl peptides cleaved from bacterial proteins, which stimulate neutrophil migration (Tonnesen et al., 1984; Bevilacqua et al., 1985; Gamble et al., 1985; Pohlman et al., 1986; Tonnesen et al., 1989).

The neutrophils primarily have an immunological function, controlling local debridement of devitalised tissue by destroying contaminating bacteria via phagocytosis and subsequent enzymatic and oxygen radical mechanisms (Schilling, 1976; Tonnesen et al., 1988; Elsbach and Weiss, 1992; Klebanoff, 1992).
Neutrophil activation by chemoattractants also stimulates release of proteolytic enzymes, such as elastase and matrix metalloproteases (MMPs). These enzymes facilitate cell penetration through blood vessel basement membranes and break down type I collagen, fibrin network, and tissue debris to prepare them for phagocytosis by macrophages (Section-1.4.2) (Horowitz et al., 1977; Clark, 1996). Neutrophil infiltration peaks at 24-48 hours postwounding, and slowly recedes as monocytes are converted into macrophages as they continue to destroy bacteria and debride the wound.

1.2.3.- Macrophages

Activation of macrophages has fundamental implications in several aspects of wound healing, such as debridement, matrix synthesis, and angiogenesis (Witte and Barbul, 1997). Circulating macrophages are stimulated by chemoattractants, these factors include fragments of collagen (Postlethwaite and Kang, 1976), elastin (Senior et al., 1980), fibronectin (Clark et al., 1988), enzymatically active thrombin (Bar-Shavit et al., 1983) and TGF-β (Wahl et al., 1987).

Macrophages release growth factors that initiate granulation tissue formation, and have the ability to continually synthesise and secrete growth factors and other cytokines (Clark, 1996). The secretion of growth factors such as TGF-β stimulates the proliferation of fibroblasts as well as increasing collagen synthesis (Roberts et al., 1986). Additionally, cytokines such as PDGF, interleukin-1, transforming growth
factor-α, insulin growth factor-1, fibroblast growth factor, and tumour necrosis factor are released from macrophages (Table 1.2.1) (Dinarello, 1984; Baird et al., 1985; Shimokado et al., 1985; Assoian et al., 1987; Madtes et al., 1988; Rappolee et al., 1988; Shaw et al., 1990, 1991).

1.3 - PROLIFERATIVE PROCESS

In full thickness wounds, connective tissue deposition, especially collagen crosslinking, is the major process responsible for repair of the damaged dermis, and the key cell responsible during this phase is the fibroblast. Proliferation of endothelial cells occurs as neovascularisation proceeds (Mast, 1992), and a viable epithelial barrier is established by the mobilisation of epithelial cells and is facilitated by the rich milieu of growth factors present within the wound (Kirsner and Eaglstein, 1993; Sempowski et al, 1995).

1.3.1.- Fibroblasts

The fibroblast is the central cell in the wound repair and scarring processes. It performs a variety of significant roles- actively producing growth factors and cytokines, degrading and synthesizing ECM proteins, and finally taking on a contractile phenotype to close the wound (Khaw et al., 1994; Sempowski et al, 1995) (FIG 1.3.1.).
FIGURE 1.3.1.- The central role of fibroblasts during wound healing
1.3.1.1.- Fibroblast- Activation

Beginning approximately 3-4 days post wounding, fibroblast infiltration and proliferation are prominent. This is mainly in response to the secreted growth factors that affect the migration and activity of fibroblasts (Mast, 1992).

The quiescent undifferentiated mesenchymal cell, the fibrocyte, converts to the active fibroblast capable of carrying out the various cellular functions responsible for wound healing when stimulated by various environmental factors. For instance, factors known to stimulate fibroblast migration are the complement component C5a (Postlethwaite et al., 1979), growth factors such as PDGF, TGF, and FGF (Postlethwaite et al., 1987), elastin and collagen fragments (Postlethwaite et al., 1976; Postlethwaite et al., 1978), leukotrienes, and fibronectin (Postlethwaite et al., 1981). These factors are derived from damaged tissue, blood, inflammatory cells (macrophages and lymphocytes), and from fibroblasts themselves (Khaw et al., 1994). Regardless of their exact origin, cytokines generated at the wound site act together to induce fibroblast proliferation and migration into the wound space and ECM production (Clark, 1996).
1.3.1.2.- Fibroblast- Migration

Cellular migration into the wound is guided by the provisional fibronectin matrix that exists within the wound, anatomical tissue planes, and planes aligned according to the tension across the wound (Schilling, 1976; Mast, 1992; Kirsner and Eaglstein, 1993).

1.3.1.2.1.- Integrins

As fibroblasts migrate into the wound space they initially penetrate the blood clot composed of fibrin and lesser amounts of fibronectin and vitronectin. Fibroblasts bind to fibronectin, vitronectin, and fibrin through specialised cell membrane receptors of the integrin superfamily (Ruoslahti, 1991; Hynes, 1992; Yamada et al., 1996; Horwitz, 1997; Witte and Barbul, 1997).

The Arg-Gly-Asp-Ser (RGDS) tetrapeptide within the cell-binding domain of these proteins is critical for binding to the integrin receptors. The integrins consist of a heterodimer of two protein chains or subunits, an α and β subunit. The α subunit is known to have about 15 variants, and the β subunit has about 8 variants. The chains which are generally named by a number or letter, combine into at least 20 different integrins (Horwitz, 1997).

Certain integrins adhere only to a single type of target molecule but others recognise multiple targets, and most interact with the ECM. These receptors recognise the ECM
and transmit signals into the interior of the cell that cause cytoskeletal reorganisation, changes in cell shape, and changes in the transcription, translation and secretion of proteins (Akiyama et al., 1990; Damsky and Werb, 1992; Faassen et al., 1992; Ginsberg et al., 1992; Hynes, 1992; Yamada et al., 1992; Juliano and Haskill, 1993; Sastry and Horwitz, 1993; Gailit and Clark, 1994; McCarthy et al., 1996; Horwitz, 1997). In addition, they help to integrate many of the diverse signals that impinge on cells through signal transduction pathways, by relaying messages through the cytoplasm (Horwitz 1997).

Fibroblasts change their integrin receptor pattern during healing (Clark, 1993). During the initial phase, a pattern promoting cell migration dominates, using mainly fibronectin and vitronectin receptors $\alpha_5\beta_1$, $\alpha_4\beta_1$ and $\alpha_6\beta_5$, while the later pattern favours cell attachment and matrix synthesis, using collagen receptors $\alpha_1\beta_1$ and $\alpha_2\beta_1$. The integrins $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are expressed mainly by fibroblasts, and mediate collagen-dependent adhesion, migration, and gel contraction and are critical for matrix remodelling after injury (Schiro et al., 1991; Dalton et al. 1995; Gotwals et al., 1996; Witte and Barbul, 1997).

TGF-β, PDGF and tumour necrosis factor-α can stimulate fibroblasts to migrate and up-regulate integrin receptors (Seppa et al., 1982; Senior et al., 1985; Postlethwaite et al., 1987; Heino et al., 1989; Clark, 1993; Ezoe and Horikoshi, 1993; Ahlen and Rubin, 1994). Therefore, expression of integrins and the migratory phenotype of
fibroblasts is possibly regulated by cytokines present at the wound sites (Clark, 1996, Witte and Barbul, 1997).

1.3.1.2.2.- Mechanisms

Fibroblasts move toward a chemotactic gradient by extending lamellipodia toward the stimulus, while their opposite pole remains firmly bound until released. This kind of cellular reorganisation is similar to fibroblast movement up a surface bound adhesion gradient, a process called haptotaxis (Carter, 1970; Harris et al., 1980; Trinkaus, 1984).

In the absence of an adhesion gradient, ECM fibrils strongly influence the direction of fibroblast migration, since cells tend to align and migrate along discontinuities in substrata to which they are attached, a process called contact guidance (Trinkaus, 1984). Cultured fibroblasts plated on preformed fibronectin fibrils have been shown to migrate along the fibrils (Hsieh and Chen, 1983; Brown et al., 1997; Wojciak-Stothard et al., 1997). Thus, chemotactic, haptotactic, and contact guidance signals may all influence fibroblast migration into the provisional matrix-filled wound space (Clark, 1996).

Movement into a cross-linked fibrin clot requires an active proteolytic system that can cleave a path for migration. A variety of fibroblast derived enzymes in conjunction with serum derived plasmin, including plasminogen activator, and matrix
metalloproteinase-1 (MMP-1), MMP-2 and MMP-3 are required (Grant et al., 1987; Wilhelm et al., 1987; Saus et al., 1988; Nagase, 1997) (Section-1.4.2.). Clot lysis is controlled by the release of plasminogen activator, which initiates lysis through conversion of plasminogen to plasmin (Loskutoff and Edgington, 1977).

The plasminogen activator type mainly responsible for clot lysis is tissue-type plasminogen activator (tPA) (Dano et al., 1985). Plasmin has the ability to degrade a wide variety of ECM proteins, however, specific inhibitors of plasminogen activator bind to the ECM and limit matrix degradation to the microenvironment around the cell surfaces (Salonen et al, 1989). Chemotactic factors, such as PDGF and TGF-β, can stimulate the production and secretion of these proteinases (Laiho et al., 1986). Once fibroblasts have migrated into the wound, they gradually switch their major function to protein synthesis (Welch et al., 1990).

1.3.1.3.- Fibroblast- Collagen Synthesis

Granulation tissue matrix deposition occurs in an ordered sequence of fibronectin, type III collagen, and type I collagen (Kurkinen et al., 1980). The entry of fibroblasts into the wound is crucial as they secrete fibronectin, glycos-aminoglycans and tropocollagen. Tropocollagen is enzymatically cross linked to form collagen (Khaw et al., 1994). Fibronectin provides a scaffold for fibroblasts to deposit collagen fibrils and begin forming granulation tissue (Woodley et al., 1985).
Collagen is the primary structural component of the repaired tissue. The production of collagen is an elaborate process involving several intracellular and extracellular events (Jackson, 1977). The deposition of collagen into the wound is coincident with the degradation of proteoglycans. Collagen is the final and permanent component of the wound matrix. The collagen molecule is composed of three polypeptide chains arranged in a right handed triple helix (Tahery and Lee, 1989; Clark, 1996). At present there are 19 collagen types designated type I-XIX according to their chronological order of discovery (Witte and Barbul, 1997).

Type III collagen is synthesised and deposited as the initial form of collagen that fills healing wounds. It provides elasticity required for increased cellularity within the healing wound, but, is quickly replaced by type I collagen, the mature and predominant collagen of skin (Gabbiani et al., 1976; Jackson, 1977; Tahery and Lee, 1989).

As collagen is the main structural protein of connective tissue it provides tensile strength for organs such as skin, bone and healed wounds. An increase in tensile strength has been correlated with an increase in the hydroxyproline content in wounds (Sandberg and Zederfeldt, 1963). In addition to fibroblasts, collagen is produced by smooth muscle cells, epithelial cells and endothelial cells (Stevenson and Mathes, 1988).
1.3.1.4.- Fibroblast-Contraction

Fibroblasts play an important role in the process of wound closure through contraction by bringing together the open margins of the wound (Gabbiani et al., 1971; Tahery and Lee, 1989; Darby and Gabbiani, 1990; Grinnell, 1994). Wound contraction is the process by which the area of tissue loss in an open wound decreases by a concentric reduction in the size of the wound (Mast, 1992).

The mechanisms of contraction during wound healing are not completely understood. However, two theories have been proposed. The first suggests that fibroblast locomotion within the connective tissue induces wound contracture (Rudolph et al., 1992). Fibroblasts generate the forces necessary for contraction by reorganising the ECM, i.e. causing collagen bundling and reorganisation. Thereby, contraction occurs as a result of tractional forces applied to the collagen substrate during movement (Harris et al., 1980; Stopak and Harris, 1982; Grinnell and Lamke, 1984; Ehrlich and Rajaratnam, 1990).

The second proposes myofibroblasts are responsible for tissue contraction (Majno et al., 1971; Rudolph, 1980; Rudolph et al., 1992; Tomasek et al., 1992; Grinnell, 1994). Myofibroblasts have large intracellular bundles of actin-micro filaments, resembling stress fibres. These cells are characterised by fibronexus-like transmembrane association between actin microfilaments and fibronectin fibrils present at their surfaces. Although, myofibroblasts express α-smooth muscle actin, these cells are
derived from fibroblasts not smooth muscle cells (Eddy et al., 1988; Darby and Gabbiani, 1990).

Based upon their spatial and temporal distribution, and the presence of large bundles of actin microfilaments, myofibroblasts have been proposed to be contractile and responsible for the production of tension in tissues undergoing contraction. Forces generated by myofibroblasts are transmitted to other cells and surrounding connective tissue, enabling these cells to act as multicellular units to contract the tissue (Gabbiani et al., 1971; Ryan et al., 1974; Rudolph et al., 1992; Gabbiani, 1992).

Wound contraction is now regarded to be caused by the active tractional forces of fibroblasts in the ECM. Actin filaments, the contractile protein of fibroblasts, exert mechanical forces on the ECM at the point of cellular attachment. Consequently, these filaments are responsible for changes in cell morphology during cell adhesion and ECM contraction, and eventually the ECM contracts and the surface area of the wound is reduced. During wound contraction, $\alpha_1\beta_1$, $\alpha_2\beta_1$, and $\alpha_3\beta_1$ integrins provide a link between fibroblasts and the ECM, allowing tensile forces generated by fibroblasts to be transmitted over the breadth of the wound (Welch et al., 1990; Schiro et al., 1991).
1.3.1.5.- Fibroblast- In vitro contraction models

A number of in vitro techniques have been used to measure the regulation of mechanical forces generated by cells. Harris et al. (1980) found that fibroblasts elastically distorted a silicone rubber substratum, due to tractional forces exerted by the cells. Tractional force measurements with cells plated onto a silicone rubber substratum have recently been modified to detect forces in the range of nano\text{Newtons} to micro\text{Newtons} (Burton and Taylor, 1997). Butt et al. (1995) and Butt and Bishop, (1997) have applied defined mechanical loads to cell monolayers, either as a static or cyclical loads (using Flexercell Strain Unit).

These investigations represent the interactions between cells and matrix in two dimensional systems. However, three dimensional (3-D) models are necessary to provide (I) spatially organised support for cells and (II) more physiologically relevant results (Eckes et al., 1995). Any material capable of forming polymer structures into which cells can attach are potential biomaterials for a 3-D model system. Agarose and other non-physiological matrices have been used, including synthetic polymers and alginate (Schlumberger et al., 1989; Fleischmajer et al., 1991). However, many models consist of type I collagen, as this is the main extracellular protein during healing.

Collagen can be extracted from several connective tissues under non-denaturing conditions, extensively purified, or used as a crude extract (Bell et al., 1979). Additionally, the collagen matrix can be modulated by changing the composition of
the macromolecules, i.e. the ratio of the different collagen types (Ehrlich, 1988a) or adding proteoglycans such as fibronectin, fibrin, and heparin (Guidry and Grinnell, 1987; Cidadao, 1989; Docherty et al., 1989; Gillery et al., 1989). These modulations influence the macromolecular organisation of the fibres and can regulate some cellular functions (Eckes et al., 1995).

The first association of fibroblasts with hydrated collagen lattices was studied by Ehrmann and Gey in 1956, followed by Elsdale and Bard in 1972. However, the association of these two components as an in vitro dermal equivalent and model for contraction was developed by Bell et al. in 1979. 3-D fibroblast populated collagen lattices (FPCLs) act as an in vitro model of ECM remodelling/reorganisation have been used to study cell-collagen interactions.

Currently, there are three variations of this model (Grinnell, 1994)- (a) free floating FPCLs in which contraction produces a reduction in diameter of the lattice, and tension is distributed isotropically and largely unopposed, (b) tethered FPCLs in which contraction reduces the lattice thickness, and tension is distributed anisotropically resulting in a stressed matrix, (c) stress relaxation with mechanical stress developing during the contraction period when the matrix is tethered, but dissipating rapidly on lattice release.
Contraction of free floating collagen matrices gives rise to a mechanically relaxed tissue, whose cells have morphological and proliferative features resembling the dermis, whilst, tethered matrices develop into a stressed tissue, resembling granulation tissue (Grinnell, 1994). The rate of contraction within collagen lattices is directly proportional to the number of cells, inversely related to the concentration of collagen, and dependent on the presence of foetal calf serum (FCS) and the integrity of the cytoskeleton (Bell et al., 1979). Fibroblasts cultured within collagen lattices actively contract the collagen lattice to approximately 10% of its original volume (Bell et al., 1979).

Morphological and physiological changes, mediated by integrins, occur to fibroblasts during contraction. Cells become elongated and bipolar, adhesion plaques are lost and the cytoskeletal elements are rearranged (Bell et al., 1979; Bellows et al., 1981; Bellows et al., 1982; Stopak and Harris, 1982; Bauer et al., 1985; Gullberg et al., 1990; Mainardi, 1993). Additionally, collagen synthesis is decreased to 10% of control values and MMP-1 gene expression is up-regulated by 30 fold resulting in collagen degradation (Mauch et al., 1988, 1989).

These models have proved to be extremely useful as they have enabled cell-matrix interactions to be demonstrated. For example, the adhesive interactions between cells and collagen required for contraction were shown to be mediated by $\alpha_2\beta_1$ integrins (Schiro et al., 1991; Klein et al., 1991), tensile force to be dependent on an intact actin...
cytoskeleton (Bell et al., 1979; Bellows et al., 1982; Guidry and Grinnell, 1985) and myosin light chain activity (Ehrlich and Griswold, 1984; Van Bockxmeer et al., 1984; Ehrlich et al., 1991). Also using these models it was found that collagen matrix contraction requires serum (Steinberg et al., 1980; Guidry and Grinnell, 1985; Tomasek et al., 1992), growth factors and cytokines influence matrix contraction with TGF-β and PDGF stimulating matrix contraction (Montesano and Orci, 1988; Clark et al., 1989; Finesmith et al., 1990; Fukamizu and Grinnell, 1990), whereas FGF and IFN-γ inhibited matrix contraction (Finesmith et al., 1990; Gillery et al., 1992). These models have enabled cell proliferation studies (Saber et al., 1981; Nishiyama et al., 1989; Nakagawa et al., 1989a and 1989b; Kono et al., 1990a) and changes in collagen and MMP biosynthesis to be examined (Nusgens et al., 1984; Unemori and Werb, 1986; Paye et al., 1987; Mauch et al., 1988, 1989; Fukamizu and Grinnell, 1990; Lambert et al., 1992; Eckes et al., 1993).

While these models enable many features of contraction to be investigated, they are however disadvantaged, since (I) mechanical forces are not measured directly, (II) the nature of mechanical loading on cells is complex and obscure, and (III) the type and magnitude of the load cannot be experimentally manipulated.

The only in vitro quantitative studies reported to measure mechanical forces have been by Kasugai et al. (1990), Delvoye et al. (1991), Kolodney and Wysolmerski, (1992), Eastwood et al. (1994, 1996, 1998), Cacou et al. (1996), and Brown et al. (1998).
These investigations use electrical strain gauges as force transducers to measure the overall isometric tension generated by a population of fibroblasts cultured within collagen matrices. These studies have shown that the force exerted by fibroblasts in tethered collagen matrices were comparable with that generated in contracting skin wounds or during tooth eruption. Recently, Brown et al. (1998) and Eastwood et al. (1998), have demonstrated that the type and magnitude of external mechanical stimulation could be modified by installing a computer controlled micro-driver to the force measurement device.

The Culture Force Monitor (CFM) and tensioning-Culture Force Monitor (t-CFM) were designed by Prof. McGrouther, Dr. Brown and Dr. Eastwood at University College London and have been reported in detail in Eastwood et al. (1994, 1996 and 1998) and Brown et al. (1998). These instruments have been used to measure the forces generated by cellular activity during FPCL contraction. Both instruments enable precise uni-axial mechanical loads to be applied to the FPCL, whilst simultaneously recording the total mechanical load across the FPCL (Section-2.2.2). Studies using the CFM have indicated that cells respond in a rapid and subtle manner to changes in tension within their matrix (Eastwood et al., 1994).

The t-CFM was developed for quantitative and directional manipulation of FPCLs by mechanical forces (FIG 2.3). It enables physiological loads to be applied across FPCLs in culture. Displacements (changes in tension) of known pattern and
magnitude, can be applied across FPCLs, and simultaneously measuring the force across that substrate. By using this instrument, it has been possible to characterise cellular responses by (I) cell-mediated changes in matrix tension, and (II) biochemical changes in cellular activity immediately following the applied mechanical stimulation. The loading regimes applied across FPCLs can be extrapolated into in vivo situations, such as those observed during skeletal muscle movement (i.e. cyclical loading regimes) and tension around dermal scars (i.e. continuous loading regimes).

All of these models allow measurements of tension generated by a population of fibroblasts cultured within collagen matrices. However, a drawback of all these models is that it is not possible to focus on the mechanical activity of individual fibroblasts within the collagen matrix, and quantitatively correlate the force generated by individual fibroblast processes.

1.3.2.- Endothelial cells.

Endothelial cells proliferate from intact venules close to the wound and form new capillaries by the process of angiogenesis (Witte and Barbul, 1997). In wound healing, angiogenesis immediately follows the movement of fibroblasts into the wound (Tahery and Lee, 1989). Angiogenesis is a complex process depending on at least four interrelated phenomena- cell phenotype alteration, chemoattractant driven migration, mitogenic stimulation, and an appropriate ECM (Clark, 1996).
1.3.3.- Epithelial cells.

Epithelisation occurs immediately after wounding, and is characterised by the movement and proliferation of epithelial cells into the wound (Tahery and Lee, 1989). The role of epithelial cell proliferation is to re-establish a barrier against fluid losses and infections, cells start proliferating, a few days after wounding, from wound edges or uninjured epithelial islands within the wound (Witte and Barbul, 1997).

1.4 - REMODELLING PROCESS

After the resolution of the initial injury the remodelling phase of the wound healing process begins. The principal processes occurring during this phase are the dynamic remodelling of collagen and the formation of the mature scar (Mast, 1992). Matrix remodelling represents a complex and dynamic balance among the forces of matrix macromolecular synthesis, degradation, and protease inhibition (Stricklin and Nanney, 1994).

This phase can occur over a number of weeks to years and is characterised by fine-tuning strength and composition of the ECM by fibroblasts (Levensen et al., 1965; Gabbiani et al., 1976; Kirsner and Eaglstein, 1993). Clinically this is the most important phase of healing because the rate, quality, and total amount of matrix deposition determine the strength of the scar. Many healing deficiencies become clinically apparent secondary to poor collagen deposition, although the underlying cause may vary, for instance, poor matrix deposition in diabetes is due in part to
reduced inflammation, whilst, excessive collagen synthesis occurs in keloid and hypertrophic scars (Section -1.5) (Witte and Barbul, 1997).

The ECM components serve several critical functions for effective wound repair. Fibronectin provides a provisional substratum for the migration and ingrowth of cells. The presence of large quantities of highly hydrated hyaluronic acid in granulation tissue provides a matrix that is easily penetrated by ingrowing parenchymal cells. The formation of types I, III and V collagen fibrils provides initial tensile strength for the wound. As the matrix matures over the following weeks, the fibronectin and hyaluronic acid disappear, collagen bundles grow in size, intensifying wound tensile strength, and proteoglycans are deposited, increasing wound resilience to deformation. Thus, early in granulation tissue formation, fibroblasts deposit a fibronectin and hyaluronan matrix that is conducive to cell migration and proliferation, and later a collagen and proteoglycan matrix that increases tissue tensile strength and resilience (Clark, 1996).

1.4.1. - Collagen remodelling during healing

Critical to the remodelling process is the conversion of the immature or provisional matrix, rich in type III collagen matrix, to a mature matrix, predominantly of type I collagen. This process requires removal of existing collagen through the action of MMPs under the control of tissue inhibitors of matrix metalloproteinases (TIMPs) produced by fibroblasts (Bailey et al., 1973; Kanzler et al., 1986).
During remodelling, the tensile strength of the wound continues to increase despite a reduction in the rate of collagen synthesis (Madden and Peacock, 1968). This gain in strength is a result of structural modifications of the newly deposited collagen, rather than its continual deposition. Covalent cross-linking which stabilises newly deposited collagen fibrils during this process is mediated by the enzyme lysyl oxidase (Jackson, 1977; Stevenson and Mathes, 1988) and provides increased strength to the wound.

Scar maturation occurs for months to years after the initial synthesis of collagen by fibroblasts. However, the repair is imperfect since the scar tissue collagen never achieves the strength or appearance of uninjured dermal collagen. The increase in tensile strength of rat wounds plateaus approximately three months following injury, reaching an apparent maximum tensile strength of 80% measured at one year postwounding in comparison with normal skin (Levenson et al., 1965).

Collagen remodelling during the transition of granulation tissue to mature scar is dependent on both continued collagen synthesis and collagen catabolism. The degradation of wound collagen is controlled by a variety of collagen degrading enzymes from granulocytes, macrophages, epidermal cells, and fibroblasts (Clark, 1996).
1.4.2. - Matrix modifying enzymes.

Proteolytic degradation of the ECM is an essential feature of repair and remodelling (Clark, 1996). The enzymes involved in the degradation of ECM macromolecules in connective tissues are proteinases. Proteinases cleave internal peptide bonds of proteins, they may be found intracellularly in lysosomes acting upon proteins taken up by endocytosis, or extracellularly in the pericellular space, and at a distance from the cells of origin (Werb and Alexander, 1993).

Four classes of proteinases have been categorised by their catalytic mechanisms: Aspartic and Cysteine proteinases are mostly active at acid pH. For instance cathepsins D, B and L, are thought to be responsible for intracellular proteolytic activity (Cawston, 1995). Serine proteases such as the plasminogen activators- urokinase-type-plasminogen activator (uPA) and tissue-type-plasminogen activator (tPA), and Matrix metalloproteinases (MMPs)- MMP-1 (collagenase), MMP-2/-9 (gelatinase-A/-B), MMP-3 (stromelysin), and membrane type matrix metallo-proteinases (MT-MMP), are active at approximately neutral pH and are responsible for extracellular digestion.

Proteolytic enzymes from all four classes have been implicated in ECM turnover (Kleiner and Stetler-Stevenson, 1993; Werb and Alexander, 1993), although MMPs and serine proteinases play a central role in inflammation, granulation tissue formation, matrix formation, and tissue remodelling. These enzymes are involved in the dissolution of the fibrin clot, ECM degradation, ECM-bound growth factor release, the
mobilisation of cells and their activation and migration into the wound area, angiogenesis, and re-epithelisation (Mignatti et al., 1996).

1.4.2.1.- Matrix Metalloproteinases (MMPs)

MMPs comprise of a family of related enzymes that share common important properties, such as their catalytic mechanism requires an active Zn$^{2+}$ site, they are secreted as latent zymogens, and have a broad substrate specificity. Various MMPs share a high degree of structural similarity, expression is tightly regulated, they are produced by many cell types, and are inhibited by tissue inhibitors. Currently 18 members have been identified (Table 1.4.1.) (Matrisian, 1990; Woessner, 1991; Birkedal-Hansen et al., 1993; Murphy and Reynolds, 1993; Werb and Alexander, 1993; Twining, 1994; Bullen et al., 1995; Mignatti et al., 1996; Nagase, 1997), which upon activation, MMPs as a group are capable of degrading all major ECM components such as collagens, proteoglycans, elastin, laminin, fibronectin and other glycoproteins (Nagase, 1997).

MMPs are inhibited by $\alpha_2$-macroglobulin and specific cell secreted proteins called Tissue Inhibitors of Matrix Metalloproteinases (TIMP-1, TIMP-2, TIMP-3, TIMP-4). The TIMPs bind to the active sites of MMPs non-covalently, but with high affinity, resulting in an inactive, stoichiometric proteinase-inhibitor complex (Howard and Banda, 1991; Bullen et al., 1995; Nagase, 1997). The balance between the activated MMPs and TIMP regulates ECM degradation (Zeng and Millis, 1994).
TIMPs have several roles during wound healing. The correct balance between MMPs and TIMPs is essential so that proteolysis can occur to enable matrix turnover and angiogenesis, but also to allow deposition of newly synthesised matrix components required for tissue repair. However, this balance is absent in cases where the healing process is disrupted, such as in chronic, non-healing wounds (Bullen et al., 1995).
Table 1.4.1.- Matrix Metalloproteinases (modified Nagase, 1997)

<table>
<thead>
<tr>
<th>ENZYME</th>
<th>MMP No</th>
<th>MATRIX SUBSTRATE or FUNCTION</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>COLLAGENASES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Interstitial collagenase</td>
<td>MMP-1</td>
<td>Collagens I, II, III, VII and X</td>
</tr>
<tr>
<td>Neutrophil collagenase</td>
<td>MMP-8</td>
<td>Collagens I, II, and III</td>
</tr>
<tr>
<td>Collagenase 3</td>
<td>MMP-13</td>
<td>Collagens I, II, III</td>
</tr>
<tr>
<td>Collagenase 4 (Xenopus)</td>
<td>MMP-18</td>
<td>Collagen I</td>
</tr>
<tr>
<td><strong>GELATINASES</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatinase B</td>
<td>MMP-9</td>
<td>Gelatins, collagens IV, V, XIV, aggrecan, elastin, entactin, vitronectin</td>
</tr>
<tr>
<td><strong>STROMELYSNINS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromelysin 1</td>
<td>MMP-3</td>
<td>Aggrecan, gelatins, fibronectin, laminin, collagens III, IV, IX and X, large tenascin-C, vitronectin</td>
</tr>
<tr>
<td>Stromelysin 2</td>
<td>MMP-10</td>
<td>Aggrecan, fibronectin, collagen IV</td>
</tr>
<tr>
<td>Stromelysin 3</td>
<td>MMP-11</td>
<td>Weak activity on fibronectin, laminin, collagen IV, aggrecan, gelatins</td>
</tr>
<tr>
<td><strong>MEMBRANE TYPE MMPs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>MMP-14</td>
<td>Collagens I, II, III, fibronectin, laminin, vitronectin, dermatan sulphate proteoglycan: activates latent MMP-2 and latent MMP-13</td>
</tr>
<tr>
<td>MT2-MMP</td>
<td>MMP-15</td>
<td>Not known</td>
</tr>
<tr>
<td>MT3-MMP</td>
<td>MMP-16</td>
<td>Activates latent MMP-2</td>
</tr>
<tr>
<td>MT4-MMP</td>
<td>MMP-17</td>
<td>Not known</td>
</tr>
<tr>
<td><strong>OTHERS</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Matrilysin</td>
<td>MMP-7</td>
<td>Aggrecan, fibronectin, laminin, gelatins, elastin, collagen IV, entactin, small tenascin-C, vitronectin</td>
</tr>
<tr>
<td>Metalloelastase</td>
<td>MMP-12</td>
<td>Elastin</td>
</tr>
</tbody>
</table>

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1.4.2.2. - MMPs - Involved in wound healing

A number of cell types produce MMPs including dermal fibroblasts (Bauer et al., 1985; Salo et al., 1985) and gingival fibroblasts (Overall et al., 1989; Salo et al., 1991), endothelial cells (Kalebic et al., 1983), osteoblasts (Rifas et al., 1989), polymorphonuclear leukocytes (Hibbs et al., 1985; Murphy et al., 1989), macrophages (Mainardi et al., 1984) and keratinocytes (Woodley et al., 1987; Salo et al., 1991; Salo et al., 1994).

Low levels of MMP activities are detected in normal steady state tissues (Nagase and Okada, 1996). MMPs are not expressed constitutively in vivo, except MMP-2, but rather are induced in response to cytokines, growth factors, hormones, oncogenes, and changes in cell contact with ECM or other cell types. Regulation of MMPs is cell type specific, for instance TGF-β stimulates MMP production in keratinocytes (Salo et al., 1991), but inhibits expression by fibroblasts, though it probably stimulates MMP-2 synthesis by fibroblasts (Edwards et al., 1987). The major inducers of fibroblast MMP expression are pro-inflammatory mediators, such as interleukin-1 and tumour necrosis factor-α (Mauviel, 1993).

MMPs are secreted from cells or expressed as plasma membrane bound forms. They have important roles in embryo development, morphogenesis, angiogenesis, tissue involution, differentiation, wound healing and in diseases associated with an unbalanced degradation of ECM. These include arthritis, glomerulonephritis,
atherosclerosis, tissue ulceration, periodontal disease, fibrotic lung disease and cancer cell invasion and metastasis (Zeng and Millis, 1994; Woessner, 1994; Nagase, 1996).

The spatial and temporal control of proteolysis is of critical importance during healing. Both MMPs and TIMPs when expressed by cells in the wound environment are not released indiscriminately, but rely on precise cell-matrix interactions to accurately remodel adjacent connective tissue.

Keratinocytes degrade dermal collagen, using MMP-1, to aid migration and promote re-epithelisation. MMP-3 and MMP-10 activity affects tissue remodelling associated with granulation tissue and scar formation. However, in chronic wounds, excessive synthesis of MMP-1, MMP-3 and MMP-10 may impair healing by destroying newly deposited matrix and cytokines, and by disrupting cell-cell interactions (Mignatti et al., 1996).

1.4.2.3.- MMP Activation

MMPs are regulated within cells and the ECM by multiple mechanisms. The mechanism controlling a given MMP depends upon the MMP itself, its particular form (latent, active or modified), and its location within a tissue. MMP synthesis is controlled at the level of gene transcription, mRNA stability, and/or translation (FIG 1.4.1).
FIGURE 1.4.1.- An overview of proteinase control mechanisms.
(modified Twining, 1994)
MMPs are synthesised as latent molecules that are activated by a conformational change and/or proteolytic cleavage. Often activation involves a cascade or amplification system in which intermediate partially activated forms are produced, culminating in the fully active form of the MMP. Additionally, MMP activity is controlled by pH, post-translational modifications (phosphorylation, glycosylation, or oxidation), localisation (in secretory granules or lysosomes or on membranes), reaction with activators or inhibitors, and proteolytic degradation (Twining, 1994).

1.4.2.4.- Activation of MMP-1 and MMP-3

The activation of the proteinases involves a number of mechanisms, which are shown to be inter-linked with each other. All MMPs are synthesized as pre-latent MMPs, and are released from the cells as latent MMPs. Latent MMP-1 and latent MMP-3 progress through step-wise activation, known as the “Cysteine switch”, which involves the dissociation of the Zn\(^{2+}\)-Cys interaction. The dissociation of the Zn\(^{2+}\)-Cys interaction allows the Zn\(^{2+}\) to interact with water, a requirement for catalysis, this is attained by the proteolytic removal of the propeptide. Several intermediate MMPs are generated which are subsequently processed into fully active MMPs (Springman et al., 1990; Nagase, 1997). Once activated, MMP-1 is involved with collagen matrix degradation. MMP-3 is associated with proteoglycan degradation. But, is also able act on a partially activated-MMP-1 to generate a fully active-MMP-1 (Suzuki et al., 1990; Murphy et al., 1992; Murphy and Reynolds, 1993) (FIG 1.4.2.).
FIGURE 1.4.2.- Activation pathways of MMPs (modified Nagase, 1997)

**EXTERNAL STIMULATION**

- **uPAR** two chain-uPA
- tPA
- Latent MT1-MMP

**PLASMIN CASCADE**

- Plasminogen
- PLASMIN
- Self generating feed-back mechanism

**LATENT MMPs**

- MMP INTERMEDIATES
- CYSTEINE - SWITCH ACTIVATION
- ACTIVE MMP-1
- ACTIVE MMP-3

**INTRACELLULAR ACTIVATION**

- Latent MMP-2
- Membrane Type 1- MMP
- ACTIVE MMP-2
- TIMP-2-MMP-2
- TIMP-1-MMP-9
- TIMP-2-MMP-2-MT1-MMP
- TIMP-1-MMP-3

**MMP-TIMP-COMPLEX ACTIVATION**

- ACTIVE MMP-1
- ACTIVE MMP-9
- MT1-MMP
- MMP-3
1.4.2.5.- Activation of MMP-2

Currently, 2 mechanisms are known for latent MMP-2 activation, both involve activation on the cell surface with Membrane Type-MMP (MT-MMP) (Nagase, 1997).

MT-MMPs are characterised by their localisation at the cell surface, where they are anchored through a transmembrane domain located at the COOH-terminal portion of the molecule. Initially, MT1-MMP was detected in malignant cells of lung and gastric carcinomas (Sato et al., 1994; Nomura et al., 1995), leading to the proposition that MT1-MMP activated latent MMP-2 at the cancer cell surface. However, MT1-MMP transcripts were also detected in fibrocytes of colon, breast, and neck carcinomas suggesting that MT1-MMP could contribute to latent MMP-2 activation under physiological conditions.

MT-MMPs have an activation site cleavable by furin-like intracellular enzymes, and thus appear on the cell surface already activated. The latent MMP-2 through its interaction with MT1-MMP on the cell surface is activated (Nagase, 1997). Active MMP-2 is known to participate in local degradation of the matrix, this is critical for cell migration and proliferation, and is thought to be the key enzyme in basement membrane degradation (Murphy and Reynolds, 1993; Mignatti et al., 1996; Nagase, 1997).
The second mechanism involves the activation of the latent MMP-2-TIMP-2 complex. The soluble catalytic domain of MT1-MMP cleaves the Asn$^{37}$-Leu$^{38}$ bond of latent MMP-2, initiating autoactivation of MMP-2 (Lichte et al., 1996; Will et al., 1996). Currently, the molecular interactions involved in the activation of latent MMP-2-TIMP-2 complex by MT1-MMP are not known.

1.4.2.6.- Activation of MMP-9

The activation of latent MMP-9 occurs by MMP-3. MMP-3 is able to catalytically activate latent MMP-9 by sequential cleavage of the Glu$^{60}$-Met$^{61}$ and Ala$^{87}$-Phe$^{88}$ bonds (Ogata et al., 1992; Nagase, 1995). MMP-3 is also required for MMP-9 activation from the latent MMP-9-TIMP-1 complex. Activation of the latent MMP-9-TIMP-1 complex requires saturation of TIMP-1 with an active MMP. A catalytic amount of MMP-3 readily activates latent MMP-9 free of TIMP-1, but not the latent MMP-9-TIMP-1 complex, as MMP-3 is inhibited by TIMP-1. However, when the latent MMP-9-TIMP-1-MMP-3 complex is formed, the interaction between latent MMP-9 and TIMP-1 is weakened and it partially dissociates into free latent MMP-9 and TIMP-1-MMP-3 complex. To fully activate the latent MMP-9 still bound to TIMP-1, an excess of MMP-3 relative to the complex is required (Goldberg et al., 1992; Ogata et al., 1992; Okada et al., 1992; Ogata et al., 1995; Nagase, 1997).

In addition to MMP-3 activation of latent MMP-9, O’Connell et al. (1994) have shown freshly generated plasmin can activate latent MMP-9, although higher
concentrations are required for total activation. The use of cell-derived plasminogen activator activity to generate plasmin from plasminogen gives partial latent MMP-9 processing.

1.4.2.7.- Plasminogen Activators

Plasmin is a proteinase derived from its abundant precursor, plasminogen, by the action of plasminogen activators, uPA and tPA. Spatial and temporal control of plasmin activity is maintained by regulating expression of the plasminogen activators, their receptors and their inhibitors- plasminogen activator-inhibitor-1 and -2, and by inhibitors of plasmin itself, such as α2-macroglobulin and the proteinase nexin (Mignatti et al., 1996). Although not all ECM proteins are substrates for plasmin, it participates indirectly in the catabolism of many ECM proteins by activating members of MMPs.

Plasminogen activators are involved with embryogenesis, angiogenesis, ovulation, neuron growth, cell migration, wound healing, vascular injury, inflammatory injury, smooth muscle cell proliferation, tumour growth, and tumour cell invasion (Twining, 1994). However, uPA and tPA have different physiological roles, uPA mediating tissue-remodelling processes and tPA involved in clot lysis (Mignatti et al., 1996).
1.4.2.8.- Activation of Plasminogen Activators

The expression of plasminogen activator activity is regulated by complex control mechanisms that act both transcriptionally and post-transcriptionally. Plasminogen activator gene transcription is modulated by a number of agents, including tumour promoters, oncogenes, growth factors, cyclic AMP, retinoids, postaglandins, and ultraviolet light (Dano et al., 1985). Post-transcriptional control of enzyme activity occurs at different levels, these include (a) proenzyme activation, (b) enzyme focalisation on the cell membrane, and (c) interaction with specific tissue inhibitors.

The secreted latent uPA binds to a specific uPA receptor on the cell surface of fibroblasts, resulting in receptor bound-uPA being converted to an active uPA. At this stage, active uPA can activate the cell-associated plasminogen to plasmin (Mignatti et al., 1996; Andreasen et al., 1997; Nagase, 1997).

Plasmin is able to degrade many ECM proteins and is involved in activation of several MMPs. The addition of plasmin to the cysteine switch pathway partially activates latent MMP-1 and MMP-3, and is also capable of activating latent MMP-9 (O’Connell et al., 1994). The generation of plasmin through the processing of plasminogen by uPA occurs at the cell surface, suggesting that the pericellular environment may be a site, in vivo, for MMP activation.
An important feature of the plasminogen activator-plasmin modulatory system is the amplification loop resulting from plasminogen and pro-uPA activation. Trace amounts of plasmin activate pro-uPA, generating a self-maintained feedback mechanism of pro-uPA and plasminogen activation (Cubellis et al., 1986; Petersen et al., 1988). Thus, production of small amounts of plasminogen activator can result in high local concentrations of plasmin.

1.5 - ABNORMAL WOUND HEALING - KEOIDS

The contraction phenomenon associated with wound healing is of prime importance during wound closure (Rudolph et al., 1992; Germain et al., 1994). This aspect of cutaneous repair is particularly important in situations in which tissue loss has occurred. Although, wound contraction is a significant process that can be life saving, it can also be responsible for deformity and disability (Rudolph, 1980).

Abnormal cutaneous wound healing results in excessive connective tissue deposition across joints or opposing areas of skin resulting in a cosmetic and/or functional deficit due to contracture, and hypertrophic scar and keloid formation (Peacock, 1984; Mast, 1992; Murry and Pinnell, 1992; Rudolph et al., 1992; Ehrlich, 1997).

Keloids are defined as abnormal scars extending beyond the confines of the original wound that rarely regress, and tend to recur after excision. Hypertrophic scars, while raised, frequently regress spontaneously and remain within the confines of the original
wound (Murry and Pinnel, 1992; Berman and Bieley, 1995). Keloids are fibrous
growths that result from an abnormal connective tissue response to trauma,
inflammation, surgery, or burns and occasionally seem to occur spontaneously (Murry
and Pinnel, 1992). Formation is principally limited to the dermis, except for the rare
keloid of the cornea. Keloids generally appear within a year after local trauma,
presenting signs and symptoms include cosmetic disfigurement, pruritus, pain, skin
discolouration, and restriction of movement. Most frequently involved sites are the
chest, shoulders, head-neck regions, especially ear lobes, arms and upper back
(Berman and Bieley, 1995).

The over abundant deposition of collagen in keloids may result from decreased
collagen degradation. Increased levels of TGF-β may lead to reduced MMP gene
expression and elevate levels of TIMPs, consequently leading to increased levels of
collagen (Nathan and Sporn, 1991; Mauviel, 1993). However, studies have indicated
that MMP-1 activity in keloids to be normal (Milsom and Craig, 1973; McCoy and
Cohen, 1982) or increased (Cohen et al., 1973), but not decreased. The effectiveness
of MMP-1 activity may be diminished (increased TIMP levels or partial activation),
even though MMP-1 levels were not decreased, this may account for altered collagen
degradation.
1.6 - AIMS AND OBJECTIVES

Many cell types are highly sensitive to mechanical loading from their surroundings. However, the mechanisms by which the cells respond are far from clear. There are at least three different types of mechanical cues- tension, compression and shear (Brown et al., 1997). The type of mechanical cue to which a cell responds is thought to reflect their normal cell environment or function, for instance fibroblasts to tensional forces, vascular endothelial cells to shear force/pressure (Levesque and Nerem, 1985; Davies, 1995), and chondrocytes to compression (Urban, 1994; Bushmann et al., 1995).

During this investigation the link between extracellular protease production and mechanical tensioning of human dermal fibroblasts was examined, to test the hypothesis that mechanical forces influence protease production. The assumption was, the generation of endogenous contractile loads and responses to externally applied loads are closely linked, consequently protease release rates would rise and fall, mirroring the function of that protease.

For this, 3 regimes of mechanical loading have been used, each model represents a different environment. The aim using these models was to systematically investigate how specific types of mechanical loading might influence tissue remodelling.
Increases in tension are minimal if the collagen lattice is untethered, since geometric contraction of the lattice occurs. The first model used was an untethered - 'minimal load' fibroblast populated collagen lattice (FPCL) system, comparable to that used widely by previous groups (Lambert et al, 1992; Tomasek et al, 1992; Grinnell, 1994), though this was rectangular rather than circular to be consistent with the subsequent models used.

The second model used was a tethered lattice within a force measurement device - the Culture Force Monitor (CFM) (Eastwood et al., 1994 and 1996). The pattern of loading was made relatively simple by restraining the contraction in only one axis. In this case, FPCLs contracted to generate their own 'static tensile load', and the magnitude of which could be continuously monitored through the CFM.

Finally, external loading was applied to the tethered system using a motorised development, the tensioning-Culture Force Monitor (t-CFM). The selected loading patterns were of very low frequency of magnitude and within physiological range of the cells (Brown et al., 1998; Eastwood et al., 1998). The loading patterns selected were (a) Cyclical loading- over and under the endogenous contraction, (b) Continuous linear loading- above the endogenous contraction for 10 mins, 1 hour and 11 hours, and continuous unloading for 11 hours below the endogenous contraction. Additionally, the effect of changing the shape of the lattice was investigated by re-orientating the configuration of the FPCL.
Fibroblasts from pathological tissues (keloid and scar) were also subjected to these three regimes of mechanical loading.

Responses of normal and pathological fibroblasts to each defined loading regime was monitored in terms of their protease production. Protease production was determined by zymography and Western blotting, and FPCLs were immunostained.
CHAPTER 2 - MATERIALS AND METHODS

All chemicals used during this investigation, unless otherwise stated, were purchased from the Sigma Chemical Company, Poole, Dorset, U.K.

2.1 - CELL CULTURE

2.1.1.- Establishment of Fibroblast Cultures

Normal human skin was obtained from mammary reductions at UCH/Middlesex Hospitals, London and Queen Mary’s Hospital, Roehampton, London. Dermal fibroblasts were grown in vitro from explants (3-5mm²) (Burt and McGrouther, 1992), plated in 25cm² culture flasks, with 5ml Dulbecco’s Modified Eagles Medium (DMEM) (GIBCO Chemicals, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (First Link, West Midlands, U.K.), 500μg/ml streptomycin and 500units/ml penicillin (GIBCO Chemicals) and gassed with 5% CO₂ prior to incubation at 37°C.

2.1.2.- Maintenance of Fibroblast Cultures

Fibroblasts had migrated out from explants by 3-4 weeks. When each flask was approximately 80-90% confluent with fibroblasts, it was passaged 1:3 into new 175cm² and subsequently 225cm² flasks. Each flask had its media changed once a week with DMEM (supplemented with 10% FCS plus 500μg/ml streptomycin and 500units/ml penicillin), gassed with 5% CO₂, incubated at 37°C, and was passaged until required for experimentation. All fibroblasts used were between passage 4-5.

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2.1.3. - Pathological Fibroblast Cultures

Pathological tissue, scar and keloid specimens, were made available from Day Surgery Unit at Middlesex Hospital, London, by Professor McGrouther, and St Thomas’ Hospital, London, by Mr P Meagher. All tissue samples were treated as above to culture fibroblasts. Fibroblasts from pathological tissues migrated out from explants by 2-3 weeks, earlier than normal fibroblasts.

2.2. - 3-D FIBROBLAST POPULATED COLLAGEN LATTICE (FPCL) CULTURES

Cell responses were compared in 3 mechanical regimes: (1) untethered (nil-tension) FPCLs, (2) static (tethered uniaxial loading in the CFM), and (3) external loading using the t-CFM.

2.2.1.- Untethered (Free floating FPCLs)

Untethered FPCLs [5x10^6 fibroblasts in 5ml collagen gel: collagen concentration 2.28mg/ml, as described previously Eastwood et al., 1994 and 1996] were prepared by mixing 4ml of native acid soluble type I rat tail collagen (First Link) with 0.5ml of 10X strength DMEM (GIBCO Chemicals), neutralising with 5M sodium hydroxide and adding to 5x10^6 fibroblasts suspended in 0.5ml DMEM. This was poured into a standard rectangular CFM culture well (75mm by 25mm by 15mm) to form a gel (gelling time approximately 10 mins). After gelling, 15ml final volume of DMEM (supplemented with 2% FCS, 2.5μg/ml Amphotericin B (GIBCO Chemicals) and
500µg/ml streptomycin and 500units/ml penicillin) was added to the cell chamber to float the gel.

2.2.2.- Static Loading (tethered FPLCs)

The experimental model consisted of a FPLC [5×10⁶ fibroblasts in 5ml collagen gel: collagen concentration 2.28mg/ml] as described above for untethered FPCLs. The collagen-cell mixture was poured into the CFM culture well, between two collagen coated flotation bars. To give a Low Aspect Ratio lattice (LAR) (i.e. length/width) the flotation bars were attached to the long edges of the lattice. All lattices, unless otherwise stated, were cast in this manner (FIG 2.2.1 and 2.2.2). After gelling, the FPCL was tethered to the force monitor using fine wire frames and floated by addition of 15ml DMEM, supplemented as above. The same 3-D collagen gel configuration was used for FPCL in the t-CFM instrument. The force generated was recorded as gross force, and the data was recorded at the rate of one reading per second, the computer software averaged 600 readings to produce one data point as described previously (Eastwood et al 1994 and 1996) as the endogenous static load.
FIGURE 2.2.1 - Schematic diagram of Culture Force Monitor (CFM)
(modified from Talas et al., 1997)
Fibroblast populated collagen lattice in a standard CFM culture well. Stainless steel suture wire frames were placed into flotation bars, linked to the fixed point and force transducer (strain gauged beam), connected to the computer. The lattice was orientated along the long axis of the culture well, known as Low Aspect Ratio lattice (LAR) (courtesy of K. Sethi, 1998).
2.2.3. Externally Loaded FPCLs

External loading across the FPCL was applied using the t-CFM (FIG 2.2.3). Cyclical loading commenced 8 hours after the start of the experiment, i.e. at the end of the main contraction phase, each cycle was applied either above or below the endogenous contraction force generated by fibroblasts (FIG 2.2.4) (Eastwood et al., 1998; Brown et al., 1998). Each cycle, CYCLICAL OVER LOAD sequence (COL), was repeated 16 times, comprising of 4 phases in each cycle: i.e. (a) 15 mins of loading, total addition load of 120 dynes, (b) 15 mins resting period (nil movement), (c) 15 mins unloading (equal and opposite to “a”) and finally (d) 15 mins resting period. The CYCLICAL UNDER LOAD sequence (CUL) was essentially a mirror of this pattern, but with an initial reduction in load of 120 dynes. The frequency of cyclical loading was low (at 2.28 x 10^-4 Hz), but the rate of change was substantial (480 dynes/hour). Thus cyclical loading was within physiological range of the cells in the collagen gel, i.e. related to the total force generated by the cells.

On completion of each experiment, the culture media was retained for analysis of proteases by zymography and Western blotting, and the FPCL was fixed in 4% paraformaldehyde (pH 7.4) for 1 hour at 4°C, and subsequently stored in 0.1M phosphate buffer (pH 7.4) at 4°C, prior to immunohistochemistry. All tethered FPCLs were fixed under tension, to ensure that cellular morphology within the lattice remained unchanged (Tomasek et al., 1992).
On the base is a petri dish containing a FPCL and below is a computer controlled nano-drive motor attached to the culture system, which was used to apply external loading patterns across the lattice (courtesy of M. Eastwood, 1998).
FIGURE 2.2.4 - Schematic diagram of (A) Cyclical Over Loading (COL) and (B) Cyclical Under Loading (CUL) around the endogenous contraction force generated.

A

FORCE (dynes)

15 min RESTING period

15 min UP loading

15 min DOWN loading

15 min RESTING period

ONE CYCLE

Endogenous contraction force

TIME (hours)

8 9 10

B

FORCE (dynes)

15 min RESTING period

15 min DOWN loading

15 min UP loading

15 min RESTING period

ONE CYCLE

Endogenous contraction force

TIME (hours)

8 9 10
2.3 - ZYMOGRAPHIC ANALYSIS

2.3.1. Gelatin Zymography

MMP-2 and MMP-9 activity in the medium was measured by gelatin zymography (Heussen and Dowdle, 1980; Brown et al., 1990). Analysis of gelatinases involved resolution by sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE), containing gelatin as a substrate: 10% Tris-Glycine buffered gel with 0.1% gelatin (pH 8.6) (Novex gels, R&D Systems, Oxford, U.K.). Proteases within the molecular weight range of 30-200 kDa were characterised. Molecular weights of proteolytic bands were determined using commercial molecular weight standards (Sigma wide range markers, 6.5-205 kDa) and the band pattern was compared to purified MMP-2 (45, 66, and 72kDa) and MMP-9 (80, 84, and 92kDa) both used at 50ng/lane (Biogenesis Ltd, Poole, U.K.).

Total 24 hour conditioned media from each experimental FPCL model was dialysed over night at 4°C into distilled water (pH 7.0), freeze dried and reconstituted in 0.5ml phosphate buffered saline (PBS) (pH 7.0). Reconstituted conditioned media samples, containing 250µg/total protein, were mixed with an equal volume of Laemmli sample buffer (2×) containing 0.5M Tris-HCl (pH 6.8), 10% (w/v) SDS, 20% glycerol, and 0.1% bromophenol blue (Laemmli, 1970). After standing for 15 mins at room temperature, 15µl of the conditioned media and Laemmli sample buffer mixture was loaded onto the gel and electrophoresed without prior reduction for approximately 1.5 hours, at constant voltage (125v) and current (40mA), in 0.24M Tris-base, 1.92M
glycine, 0.035 M SDS, until the tracker dye reached the gel base. After electrophoresis, gels were washed in 2.5% Triton-X-100 for 30 mins at room temperature to renature proteins. Gels were washed in 50 ml developing buffer (pH 7.72, 0.1 M Tris-base, 0.4 M Tris-HCl, 2 M NaCl, 0.067 M CaCl$_2$, 0.2% w/v Brij 35) for 30 mins at room temperature, then incubated in fresh developing buffer (overnight) at 37°C, containing 2 mM p-amino-phenyl-mercuric-acetate (APMA) to activate pro-enzymes forms of MMP species present in the gels. Gels were stained with 0.1% Coomassie blue R250 (Merck, Poole, U.K.) in methanol, distilled water, glacial acetic acid (45:45:10 vol/vol) with agitation at room temperature for 2 hours. Following destaining in methanol, distilled water, glacial acetic acid solution (45:45:10 vol/vol), bands of enzyme activity could be identified from lysis of the gelatin resulting in clear bands on a blue background, indicating the presence of gelatinase (Leber and Balkwill, 1997).

### 2.3.2.- Casein Zymography

Casein gels were run in a similar manner to gelatin zymography. Gels were 12% Tris-Glycine gel with β-casein incorporated as a substrate (Novex gels, R&D Systems, Oxford, U.K.). However, for these gels 500 μg/total protein from 0.5 ml reconstituted conditioned media was used, and mixed with an equal volume of Laemmli sample buffer (2×). After standing for 15 mins at room temperature, 15 μl of the conditioned media and Laemmli sample buffer mixture was loaded onto the gel and electrophoresed as described above. Proteases within the molecular weight range of
30-200 kDa were characterised. Molecular weights of proteolytic bands were
determined by comparing to commercial molecular weight standards Sigma Marker
wide range (6.5-205 kDa) (Sigma Chemicals) and purified MMP-3 (28, 36, 57,
59kDa) 50ng/lane (Biogenesis Ltd, Poole, U.K.).

2.3.3.- Plasminogen Zymography

urokinase type plasminogen activator (uPA) and tissue type plasminogen activator
(tPA) were identified in a similar zymogram system by incorporation of 0.176 unit/ml
plasminogen from human plasma (Sigma Chemical) reconstituted in PBS and added to
casein gels (Fisher and Werb, 1995). 72mg casein (Hammersten casein, Merck) was
incorporated into 10% SDS-PAGE gels in 10ml 1.5M Tris/HCl (pH 8.8), dissolved at
60°C in a water bath. Reconstituted conditioned media samples, containing
250μg/total protein, were mixed with an equal volume of Laemmli sample buffer (2×).
After standing for 15 mins at room temperature, 15μl of the conditioned media and
Laemmli sample buffer mixture was loaded onto the gel and electrophoresed without
prior reduction for approximately 1.5 hours at constant voltage (100v) and current
(20mA), in 0.192M glycine, 0.025M Tris, and 0.1% SDS at pH 8.3. Gels were
washed in 2.5% Triton-X-100 for 30 mins at room temperature, immersed in
developing buffer (0.05M Tris-HCl, 0.5M NaCl, 0.05M CaCl₂, pH 7.0) overnight at
37°C and stained with 0.1% Coomassie blue R250 (Paul et al., 1997). Molecular
weights of proteolytic bands were determined using commercial molecular weight
standards Sigma Marker wide range (6.5-205 kDa) (Sigma Chemicals).
2.3.4.- Quantitation by Scanning Densitometry

Following destaining, zymogram gels were dried before quantitation by scanning densitometry. Each gel was placed in a drying solution of 33% ethanol, 1% glycerol in distilled water for 15-20mins, at room temperature. After which it was laid between two cellophane sheets in a plastic frame (Novex), overnight at room temperature, in an upright position. Zymograms were scanned using a Microdensitometer mark IIICS (Joyce Loebl, Gateshead-on-Tyne, U.K.) to give relative intensities of bands compared to purified enzyme standards. By including purified enzyme standards on each zymogram, the sample enzyme activities could be expressed in relation to the purified standard, thus allowing comparisons to be made between samples run on separate gels. Enzyme activity for MMP-2, MMP-3 and MMP-9 were expressed as nanograms per million cells/ hour, and as arbitrary absorption units per million cells/ hour for plasminogen activators- as no enzyme standard was used. On each occasion prior to use the Micro-densitometer was set to provide a stable base line for each zymogram, to ensure results were reproducible. The settings used were: optical wedge-B100, ratio arm-5:1, differential control-6, pen damping-7, aperture width-0.5, aperture height-2.5. The data was statistically analysed using computer software (MINITAB release 10.1 for Windows) Students t Test.
2.4 - WESTERN BLOTTING ANALYSIS

Reconstituted conditioned media samples, 250μg/total protein, were mixed with an equal volume of Laemmli sample buffer (2x) (pH 6.8) containing 10% (w/v) SDS with β-mercaptoethanol and heated to 100°C for 5 mins. 15μl of the conditioned media and Laemmli sample buffer mixture was loaded onto 8% SDS-PAGE gels for approximately 1.5 hours at constant voltage (90v) and current (40mA) in 0.625M Tris, 4.8M glycine, and 0.08M SDS. Followed by electrophoretic protein transferance to Hybond Enhanced chemiluminescence nitrocellulose membrane (Amersham Life Science, Little Chalfont, U.K.) overnight at 4°C, at constant voltage (50v) and current (100mA) in 0.025M Tris, 0.192 glycine, 20% methanol (pH 8.3). After washing for 3x 5mins in Western wash solution (1.5M sodium chloride, 0.1M Tris, 0.005% Tween-20, pH 7.4), 5% dried milk powder (Marvel™, Cadbury, Birmingham) in Western wash solution was used to block non-specific binding sites to the nitrocellulose membrane for 1 hour at 37°C. Primary antibodies used were all polyclonal sheep anti-human IgG to MMP-1, -MMP-2, -MMP-3, -MMP-9, and TIMP-1 (50μg/ml) in 2.5% dried milk powder in Western wash solution. These antibodies were all prepared and characterised as described in the following references (Hembry et al., 1985; Hembry et al., 1986; Whitham et al., 1986; Murphy et al., 1989; Allen et al., 1991; Hipps et al., 1991). All primary antibodies were kindly provided by Dr.G.Murphy and Dr.R.Hembry, Strangeways Research Laboratory, Cambridge, U.K. Incubation with primary antibody, for 3 hours at room temperature, was followed by washing 3x 5mins in Western wash solution. Incubation with 5% dried milk powder
in Western wash solution for 1 hour at 37°C was used to block non-specific binding sites. The blots were incubated with the secondary antibody a peroxidase conjugated donkey-anti-sheep-IgG (Sigma Chemicals) (1:2000 dilution) for 1 hour at room temperature. Followed by washing 3x 5mins in Western wash solution. The nitrocellulose membrane was incubated with Super Signal Substrate (Pierce and Warriner Ltd, Chester, U.K.) for 5mins (an equal volume of Luminol/Enhancer solution and Stable Peroxide solution, 0.125ml/cm² of membrane surface), and wrapped in cling film. The wrapped nitrocellulose membrane was exposed to high performance enhanced chemiluminescence detection film (Amersham).

2.5 - IMMUNOFLUORESCENT STAINING

On completion of untethered and tethered FPCL experiments lattices were fixed in 4% paraformaldehyde (pH 7.4) for 1 hour at 4°C, all tethered gels were fixed under tension. Lattices were washed in PBS (pH 7.0) for 1 hour at 4°C. Each lattice was cut into approximately 1cm² pieces. Each piece was placed in PBS with 1% Triton-100-X for 1 hour at room temperature. Followed by DMEM with 1% Bovine Serum Albumin (BSA) (Sigma Chemicals) for 1 hour at room temperature. Primary antibodies used were polyclonal sheep anti-human IgG to MMP-1, MMP-2, MMP-3, MMP-9, TIMP-1 and TIMP-2 in DMEM with 1% BSA, all at 50µg/ml. All primary antibodies were kindly provided by Dr.G.Murphy and Dr.R.Hembry at Strangeways Research Laboratory, Cambridge, U.K., and prepared and characterised as described above (Section 2.4). The negative control used was normal sheep serum (Sigma Chemicals).
The lattices were incubated with the primary antibody overnight at 4°C. Followed by 6x 1 hour washes in PBS at room temperature. Secondary antibody used was Rabbit-F(ab')2-anti-sheep-IgG-FITC (Euro-Path Ltd, Stratton, UK) (1:100 dilution) in DMEM with 1% BSA. The lattices were incubated with the secondary antibody overnight at 4°C. Followed by 6x 1 hour washes in PBS at room temperature. The lattices were counterstained with 1% propidium iodide, and mounted on microscope slides with Citifluor (Citifluor Ltd, University of Kent, Canterbury, U.K.). Lattices were viewed by epifluorescence (Leitz, M.K., U.K.) using standard wide and narrow band filters. Photographs were taken on Kodak P1600x ASA Ektachrome professional film.
CHAPTER 3- METHOD DEVELOPMENT

3.1. - SERUM CONCENTRATION AND CONTRACTION

For this study contraction of FPCLs were carried out in 2% FCS rather than the routine level of 10% FCS, to assist with subsequent protease measurements as serum contains MMPs and inhibitors to them (Section-3.4). Serum concentration levels are known to influence FPCL contraction (Tomasek et al., 1992; Grinnell, 1994), and as such the system was first tested using contracting untethered FPCLs over 24 and 48 hours, in DMEM containing 2% or 10% FCS, or 2% or 10% gelatinase/fibronectin depleted FCS, using triplicate FPCLs. Gelatinase/fibronectin depleted FCS was prepared by incubating FCS with gelatin coated Sepharose beads (Sigma Chemicals), (diluted 1:10 in normal FCS) for 2 hours at 4°C as described by Azzam et al., 1992. Area measurements of untethered FPCLs at specific time points were made by photographing the whole lattice within the CFM culture well on a light box, scanning into an Improvision Image Analysis System (Adobe photoshop 3.0.5.) and measuring using NIH Image 1.59 programme. All areas were related as a percentage to that of the CFM culture well (75mm by 25mm by 15mm), and data statistically analysed using Students t Test (computer software MINITAB release 10.1 for Windows).

Untethered FPCLs in low and high FCS (2 and 10%) contracted to 64.6 ± 0.3% and 46.3 ± 0.9%, respectively at 24 hours (FIG 3.1.1). By 48 hours this had further
reduced to 60.0 ± 0.9% and 28.7 ± 1.9% of the original area, for 2% and 10% FCS respectively.

Contraction of untethered FPCLs using 2% and 10% gelatinase depleted FCS at 24 hours was 98.4 ± 0.7% and 95.9 ± 0.2% respectively, by 48 hours this had only slightly reduced to 98.3 ± 0.4% and 92.17 ± 1.0%, respectively (FIG 3.1.2.). Control cell-free lattices placed in serum free DMEM did not contract over 24 and 48 hours. Thus, the rate of contraction was related to the percentage of FCS present within the culture media and due to the presence of fibroblasts within the lattices (FIG 3.1.3).
FIGURE 3.1.1 - Contraction of untethered FPCLs in DMEM with 2% and 10% FCS at 24 and 48 hours. (Bar = 10mm).

KEY: A and B - FPCL in 10% FCS
C and D - FPCL in 2% FCS
E and F - Control cell-free lattice in 0% FCS
FIGURE 3.1.2.- Contraction of untethered FPCLs in DMEM with 2% and 10% gelatinase depleted FCS at 24 and 48 hours. (Bar = 10mm).

KEY:  
A and B - FPCL in 10% gelatinase depleted FCS  
C and D - FPCL in 2% gelatinase depleted FCS  
E and F - Control cell-free lattice in 0% FCS

24 HOURS  
48 HOURS
FIGURE 3.1.3.- Graph of contraction of untethered FPCLs

Increasing the percentage of FCS in DMEM resulted in a significant increase in FPCL contraction with time.  $p < 0.01$ 2% and 10% FCS vs. cell/serum free control. (n=3 for each lattice)
Tethered endogenous contraction of FPCLs (endogenous static loading) using the CFM demonstrated that the average contraction force at 8 hours was 51 ± 4.5 dynes (n=3) with 2% FCS, compared with 380 ± 15 dynes (n=3) with 10% FCS, giving a percentage difference in 8 hour contraction of 87% (FIG 3.1.4.).

![FIGURE 3.1.4.](image)

**FIGURE 3.1.4.** Endogenous contraction forces generated by FPCLs in either 2% or 10% FCS. Dotted line represents 8 hours.
3.2. - STANDARD CURVE ANALYSIS OF PURIFIED ENZYME

STANDARDS AND CONDITIONED MEDIA

Standard curve analysis of purified MMP-2 and MMP-9, and those from conditioned media were investigated in order to evaluate the linearity of the zymogram. A range of concentrations of MMP-2 and MMP-9 standard from 16ng to 250ng/lane were run on zymograms. Enzyme activity for each of the latent and active bands for both MMP-2 and MMP-9 decreased proportionally with the amount of enzyme loaded (FIG 3.2.1). Conditioned media collected from an untethered FPCL contraction (with 2% FCS) was run from 4μg to 500μg/total protein. Protein concentration was measured using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hemel Hempstead, UK). Enzyme activity from the conditioned media decreased with sample loading (FIG 3.2.2). The clearest enzyme activity bands were given with 250μg/total protein loading per lane, all subsequent analysis of conditioned media on gelatin zymograms were run at this concentration. Standard curves of MMP-2 and MMP-9 had a linear relationship with enzyme loading for 16-250 ng/lane (FIG 3.2.3 and 3.2.4 respectively). FIG 3.2.5. shows the curve for latent MMP-2 from the conditioned medium sample.
FIGURE 3.2.1. - (A) Gelatin zymogram showing serial dilutions of MMP-2 and MMP-9 (from 250ng to 16ng/lane). (B) Scan of gelatin zymogram showing intensities of enzyme bands.

Key:

Lane A- MMP-2 Standard 250ng
Lane B- MMP-2 Standard 125ng
Lane C- MMP-2 Standard 63ng
Lane D- MMP-2 Standard 32ng
Lane E- MMP-2 Standard 16ng
Lane F- MMP-9 Standard 250ng
Lane G- MMP-9 Standard 125ng
Lane H- MMP-9 Standard 63ng
Lane I- MMP-9 Standard 32ng
Lane J- MMP-9 Standard 16ng
Lane K- Base line

SCAN only:

I- 72kDa Latent MMP-2
II- 66kDa Active MMP-2
III- 45kDa Active MMP-2
IV- 92kDa Latent MMP-9
V- 84kDa Active MMP-9
VI- 80kDa Active MMP-9
FIGURE 3.2.2.-(A) Gelatin zymogram showing serial dilutions of conditioned media sample (from 500µg to 4µg/total protein). (B) Scan of gelatin zymogram showing intensities of enzyme bands.

Key - ZYMOGRAM and SCAN

Lane A- MMP-2 Standard
Lane B- Conditioned media 500µg/total protein
Lane C- Conditioned media 250µg/total protein
Lane D- Conditioned media 125µg/total protein
Lane E- Conditioned media 62µg/total protein
Lane F- Conditioned media 32µg/total protein
Lane G- Conditioned media 16µg/total protein
Lane H- Conditioned media 8µg/total protein
Lane I- Conditioned media 4µg/total protein
Lane J- MMP-9 Standard
Lane K- Base line

SCAN only

I- 72kDa Latent MMP-2
II-66kDa Active MMP-2
III-45kDa Active MMP-2
IV- 92kDa Latent MMP-9
V- 84kDa Active MMP-9
VI-80kDa Active MMP-9
FIGURE 3.2.3.- Standard curve for MMP-2 (using a simple curve fit).

A linear relationship between enzyme loading and activity was detected.

- 72 kDa - Latent MMP-2
- 66 kDa - Active MMP-2
- 45 kDa - Active MMP-2

\[ y = 12002.03 + 472984.02x \quad r^2 = 0.99 \]
\[ y = 21381.36 + 444042.10x \quad r^2 = 0.98 \]
\[ y = -5311.48 + 180057.04x \quad r^2 = 0.98 \]
A linear relationship between enzyme loading and activity was detected.

\[
\begin{align*}
    y &= 3829.85 + 261554.72x \quad r^2 = 0.95 \\
    y &= -1913.47 + 77998.65x \quad r^2 = 0.98 \\
    y &= -1495.91 + 45601.20x \quad r^2 = 0.97
\end{align*}
\]
FIGURE 3.2.5. - Curve for MMP-2 from conditioned sample media

(using a polynomial curve fit).

\[ y = -0.01 + 2.38x - 0.03x^2 - 4.93x^3 \quad r^2 = 1.00 \]
3.3. - ACTIVATION AND INHIBITION OF PROTEASES

Analysis of culture media by zymography demonstrated the presence of a range of metallo- and serine proteases in latent and active forms: MMP-2, MMP-9, MMP-3, uPA and tPA. Confirmation of the identity of the protease class responsible for enzyme activity was established by separating conditioned media samples and enzyme standards (where applicable) by zymography, cutting the lanes into strips, followed by incubation in renaturing buffer and the first proteolysis buffer (described above) and finally including an activator or inhibitor for proteases in the proteolysis buffer overnight at 37°C. MMP activity was confirmed with activation by 2mM APMA and inhibition with 10 mM 1,10 phenanthroline or 50mM EDTA. APMA is an organomercurial compound that causes a confirmational change to occur within the latent enzyme allowing autocatalytic cleavage to the active form (Stricklin et al., 1983). 1,10 phenanthroline is a zinc chelator inhibiting gelatinolytic activity (Le et al., 1991; Nagase et al., 1992; Galis et al., 1995; Nagase, 1997). Specific serine protease activity was demonstrated by inhibition, with increasing concentrations of 1, 5, 10mM phenyl methyl sulphonyl fluoride (PMSF) and 1, 10, 50mM e-amino caproic acid, and for uPA 1, 5, 10mM amiloride.

MMPs are initially produced as high molecular weight latent enzymes which can be processed to give the active enzyme, at a lower molecular weight (Woessner, 1991; Matrisian, 1992). MMP activation and inhibition were demonstrated on gelatin and casein zymograms (FIG 3.3.1 and 3.3.2 respectively). On either side of the
conditioned media sample on the gelatin zymogram (FIG 3.3.1.) were placed the MMP-2 and MMP-9 standards, and on the casein zymogram (FIG 3.3.2.) the conditioned media and MMP-3 standard were run. Incubation with APMA activated the latent enzymes to their active forms, whereas incubation in only proteolysis buffer produced much less active enzyme. Incubation with the inhibitors completely blocked activity. Complete inhibition of uPA and tPA was demonstrated with increasing concentrations of PMSF and e-amino caproic acid, incubation with amiloride blocked all uPA, but not that of tPA on the plasminogen zymogram (FIG 3.3.3.). Thus, the identity of protease activity was confirmed, distinguishing the individual metallo- and serine proteases.
**FIGURE 3.3.1.-** Gelatin zymogram- Activation and Inhibition of MMP standards and conditioned medium.

**KEY**- Lane A- MMP-2 standard  
Lane B- Conditioned medium  
Lane C- MMP-9 standard

1 **MMP standards and conditioned medium in proteolysis buffer.**
MMP-2 standard activity was shown at 72kDa, 66kDa, and 45kDa, and MMP-9 activity at 92kDa, 84kDa, and 80kDa. Enzyme activity within the conditioned medium was present mostly at 72kDa, latent MMP-2, with a weaker enzyme band detected at 92kDa, latent MMP-9.

2 **Activation of MMP standards and conditioned medium in 2mM APMA.**
Following APMA incubation latent enzyme bands in the conditioned medium were fully activated, from 72kDa to 66kDa, latent and active MMP-2 respectively, and 92kDa to 84kDa, latent and active MMP-9 respectively.

3 **Inhibition of MMP standards and conditioned medium in 10 mM 1,10 phenanthroline.**
All enzyme activity in MMP standards and conditioned medium was blocked following incubation with 10mM 1,10 phenanthroline, (with only the slightest detection of MMP-2 possible).

4 **Inhibition of conditioned medium in 50mM EDTA.**
No enzyme activity was detected in the conditioned medium sample following incubation with 50mM EDTA.
1. MMP standards and conditioned medium in proteolysis buffer.

2. Activation of MMP standards and conditioned medium in 2mM APMA.

3. Inhibition of MMP standards and conditioned medium in 10 mM 1,10-phenanthroline.

4. Inhibition of conditioned medium in 50mM EDTA.
FIGURE 3.3.2.- Casein zymogram- Activation and Inhibition of conditioned media and MMP-3.

KEY- Lane A- Conditioned medium
Lane B- MMP-3 standard

1 MMP standard and conditioned medium in proteolysis buffer.
MMP-3 standard activity was shown at 59kDa. Enzyme activity within the conditioned medium was present mostly at 97 and 84kDa. Non-enzymatic protein bands (dark bands) in the conditioned medium were present above and below the enzyme bands.

2 Activation of MMP standard and conditioned medium in 2mM APMA.
Following APMA incubation the intensity of the conditioned medium enzyme bands at 97 and 84kDa increased, additionally a band was detected at 28kDa, suggesting latent enzyme activation. MMP-3 standard enzyme bands were easily observed at 59kDa, 57kDa, 36kDa and 28kDa.

3 Inhibition of MMP standard and conditioned medium in 10 mM 1,10 phenanthroline.
All enzyme activity in the MMP standard and conditioned medium was blocked following incubation with 10mM 1,10 phenanthroline.

4 Inhibition of MMP standard and conditioned medium in 50mM EDTA.
No enzyme activity was detected in the MMP standard and conditioned medium sample following incubation with 50mM EDTA.
1. Conditioned medium and MMP standard in proteolysis buffer.

2. Activation of conditioned medium and MMP standard in 2mM APMA.

3. Inhibition of conditioned medium and MMP standard in 10 mM 1,10 phenanthroline.

4. Inhibition of conditioned medium and MMP standard in 50mM EDTA.
FIGURE 3.3.3.- Plasminogen zymogram-Inhibition of conditioned media.

1 Conditioned medium in proteolysis buffer.
Enzyme activity in the conditioned medium was detected at 66kDa for tPA, and at 55kDa and 33 kDa for latent and active uPA.

2 Inhibition of conditioned medium in 1, 5, 10mM PMSF
Following incubation with increasing concentrations of PMSF all uPA and tPA enzyme activity within the conditioned medium was completely blocked.
KEY- Lane A-1mM PMSF
   Lane B- 5mM PMSF
   Lane C-10mM PMSF

3 Inhibition of conditioned medium in 1, 10, 50mM ε-amino caproic acid.
Following incubation with increasing concentrations of ε-amino caproic acid all uPA and tPA enzyme activity within the conditioned medium was completely blocked.
KEY- Lane A- 1mM ε-amino caproic acid
   Lane B- 10mM ε-amino caproic acid
   Lane C- 50mM ε-amino caproic acid

4 Inhibition of conditioned medium in 1, 5, 10mM amiloride.
Following incubation with increasing concentrations of amiloride all uPA enzyme activity within the conditioned medium was completely blocked, however tPA was not inhibited.
KEY- Lane A- 1mM amiloride
   Lane B- 5mM amiloride
   Lane C- 10mM amiloride
1. **Conditioned medium in proteolysis buffer.**

2. **Inhibition of uPA and tPA in conditioned medium following incubation in 1. 10 mM PMSF**

3. **Inhibition of uPA and tPA in conditioned medium following incubation in 1. 50 mM α-amino caproic acid.**

4. **Inhibition of uPA but not tPA in conditioned medium following incubation in 1. 5, 10 mM amiloride.**
3.4 - FETAL CALF SERUM CONTROL

3.4.1.- Presence of proteases in FCS

Variability between experiments was reduced by ensuring all experiments were performed using the same batch of FCS. FCS contains a wide range of unspecified proteases and cytokines which can influence the synthesis of MMPs by cells, including fibroblasts.

To select the FCS to use 15ml of four different batches of FCS (GIBCO) were incubated at 37°C for 24 hours. They were prepared for zymography as described above, and separated on gelatin, casein and plasminogen zymograms.

Separation by zymography revealed that all four batches of FCS did contain varying levels of protease activity (FIG 3.4.1.). Gelatin zymography showed that the predominant protease activity was latent MMP-9, followed by a weaker latent MMP-2 band. The enzyme activities for these bands are shown in FIG 3.4.2. No enzyme activity from any batch of FCS was detected by casein zymography, whereas by plasminogen zymography, enzyme activity was only present at 84 kDa, higher than the molecular weight of tPA (66 kDa) and uPA (55 kDa). Therefore, for all subsequent experiments only one batch of FCS was selected, this was FCS batch 1 (lane A) in FIG 3.4.1 and column 1 of FIG 3.4.2.
FIGURE 3.4.1.- FCS Control - on Gelatin, Casein and Plasminogen zymograms

KEY- Lane A- FCS Batch 1
     Lane B- FCS Batch 2
     Lane C- FCS Batch 3
     Lane D- FCS Batch 4

1 FCS Control - Gelatin zymogram

Proteolytic activity within FCS was present at 72kDa latent MMP-2, and 92kDa and a weaker band at 80kDa for MMP-9.

2 FCS Control - Casein zymogram

No proteolytic activity from any FCS batch was detected on the casein zymogram.

3 FCS Control - Plasminogen zymogram

FCS proteolytic activity was seen only at 84kDa.
1. FCS Control - Gelatin zymogram

2. FCS Control - Casein zymogram

3. FCS Control - Plasminogen zymogram
FIGURE 3.4.2.-Graph shows the difference in enzyme levels of MMP-2 and MMP-9 present in four different batches of FCS.

3.4.2.-Protease activity in growth media with 0%, 2%, 10% FCS.

Having selected one batch of FCS, it was added to DMEM at 0%, 2%, and 10% to determine the protease activity in the absence of cells. This level could then be subtracted from the conditioned media samples to obtain the amount of enzyme produced by the cells. 15ml of DMEM with 0%, 2%, and 10% FCS was incubated at 37°C for 24 hours and then run on gelatin, casein and plasminogen zymograms as previously described (Section-3.2).
No bands of enzyme activity were present with media alone (0% FCS) on any of the zymograms. Neither was enzyme activity observed on the casein zymogram in the presence of serum (2% and 10% FCS). For the plasminogen zymogram a band occurred only at 84kDa. However, on the gelatin zymogram protease bands were detected at 92kDa and 84kDa (latent MMP-9) and at 72kDa (latent MMP-2) with media containing 2% and 10% FCS. Thus, this gelatinase activity due to FCS was subtracted from all subsequent experimental conditioned media (FIG 3.4.3. and 3.4.4).

![Graph showing total levels of MMP-2 and MMP-9](image)

**FIGURE 3.4.3.** - Graph shows the total levels of MMP-2 and MMP-9 present in media with 0%, 2%, and 10% FCS.
FIGURE 3.4.4.- Media FCS Control- on Gelatin, Casein and Plasminogen zymograms

KEY  Lane A- 0% FCS in DMEM

        Lane B- 2% FCS in DMEM

        Lane C- 10% FCS in DMEM

1  FCS Control - Gelatin zymogram

Enzyme bands were only observed at 92kDa and 84kDa for MMP-9 and 72kDa for MMP-2 in 2% and 10% FCS only.

2  FCS Control - Casein zymogram

No proteolytic activity from FCS was detected on the casein zymogram.

3  FCS Control - Plasminogen zymogram

FCS proteolytic activity was seen only at 84kDa.
1. FCS Control - Gelatin zymogram

2. FCS Control - Casein zymogram

3. FCS Control - Plasminogen zymogram
3.5 - PERCENTAGE OF BOUND vs UNBOUND MMP IN THE COLLAGEN LATTICE AND MEDIA

The level of proteases that remained within the collagen lattice after synthesis by fibroblasts was also studied. This was investigated by using Woessner’s method for Quantitation of MMPs in Tissue samples (Woessner, 1995). This involved two extraction steps, (I) a triton extraction, which disrupted the fibroblasts within the lattice, to release intracellular and membrane bound enzyme. (II) a heat extraction step (60°C), in the presence of high calcium concentration (0.1M CaCl₂ and 0.15M NaCl in 50mM Tris-HCl, pH 7.5), which released any enzyme remaining bound to the lattice. The triton and heat extracts and the conditioned media were analysed for gelatinase activity by zymography as previously described.

Measurement of total recovered MMP-2 activity found 1.9% of MMP-2 was present in the heat extract, 7.5% in the triton extract, and 90.6% in the conditioned media (FIG 3.5.1). However, no MMP-9 was detected in either the triton or the heat extracts, but did occur in the conditioned media (FIG 3.5.2.). Thus, all of the MMP-9 and the majority of the MMP-2 produced by fibroblasts in lattices were secreted into the media and not bound to the lattice.
FIGURE 3.5.1.-Total MMP-2 levels present in lattice extracts and conditioned media. The difference in enzyme levels was significant $p < 0.001$ media vs. heat extract. ($n=3$)

FIGURE 3.5.2.-Total MMP-9 levels present in lattice extracts and conditioned media. Enzyme levels were detected only in the conditioned media. ($n=3$)
3.6 - ANALYSIS OF CONDITIONED MEDIA

Conditioned media collected from untethered FPCL contraction at 8, 24, and 48 hours (Section-3.1) were analysed by gelatin zymography, to quantify the levels of MMP-2 and MMP-9 when the lattices were placed in culture media with different percentages of FCS: 2% and 10% FCS, and 2% and 10% gelatinase depleted FCS.

For each set of experiments, for both MMP-2 and MMP-9, the greatest rate of enzyme release was at 8 hours, decreasing at 24 hours, and lower still at 48 hours. The highest enzyme activity was observed with 10% FCS in culture media. Total levels of MMP-2 and MMP-9 present in conditioned media with 2% and 10% FCS are shown in FIG 3.6.1. For MMP-2 the difference in enzyme activity between 2% (A) and 10% (B) was at 8 hours 3.2 fold, 3.3 fold at 24 hours, and at 48 hours was 2.7 fold. A similar trend was seen with MMP-9, the difference in enzyme activity between 2% (C) and 10% (D) was 7 fold at 8 hours, at 24 hours it was 2.3 fold, and by 48 hours it was 2.5 fold. Thus, increased enzyme activity was due to the level of FCS present in the culture media.

To assess the effect of gelatinase present in FCS on synthesis of further gelatinase by fibroblasts during contraction, enzyme activity in the conditioned medium was measured by zymography after contraction using gelatinase-depleted FCS at 2% and 10%. MMP-2 and MMP-9 were still present in the media although levels were not as high as in the untreated FCS. Similarly to untreated FCS maximum activity
was measured at 8 hours and levels were higher with 10% compared to 2% FCS (FIG 3.6.2).

All subsequent contraction experiments (non-loaded, static loaded, and externally loaded FPCLs) were performed in 2% FCS, as subtle changes in protease activity resulting from loading may become masked if 10% FCS were used. On separation by zymography, the enzyme levels produced by FCS in media were subtracted from all experimental conditioned media (Section 3.4).
Contracted at B, 24, and 48 hours (n=3).

Present in 2% (A) and (C) and 10% (B and D) media during unrelated FPC.

FIGURE 3.6-1. Total release rates of MNF-2 (A) and B) and MNF-2 (C and D).
FIGURE 3.6.2.-Total release rates of MMP-2 (A and B) and MMP-9 (C and D) present in 2% (A and C) and 10% (B and D) gelatinase depleted media during untethered FPCL contraction at 8, 24, and 48 hours. (n=3)
3.7. - TETHERED EXTERNALLY LOADED FPCLs

The tensioning-Culture Force Monitor (t-CFM) was developed for quantitative and directional manipulation of FPCLs by mechanical forces in culture (FIG 2.3). The system was driven via a precision ground leadscrew with a pitch of 0.508mm, by a stepper motor and controller with a resolution of up to 50800 steps/revolution, to give a reproducible position capable of $1 \times 10^{-8}$m. The loading cycle could be varied in its frequency (range between 50Hz and $1 \times 10^{-5}$Hz), and also in the amplitude and resting time between loading regimes. Thereby, the t-CFM could precisely apply external mechanical stimulation to fibroblasts within a collagen lattice in a controlled manner.

All loading programmes were written and installed onto the t-CFM by Dr. Eastwood. All loading regimes commenced at 8 hours, at the end of the main contraction period. The loading patterns selected were (a) Cyclical loads- *Over* and *Under* the endogenous contraction (Section-2.2.3), (b) Continuous linear loads- *Unloading* and *Loading* below and above the endogenous contraction force, respectively.

During the CONTINUOUS UNLOAD sequence (FIG 3.7.1.), the micro-driver was programmed to *unloading* the FPCL for 11 hours (total unloading of 480 dynes), i.e. continuously unloading below the endogenous contraction force generated by fibroblasts. The CONTINUOUS LOAD sequence was the opposite, by *loading* the FPCL for 10 mins (total load of 4000 dynes), 1 hour (total load of 480 dynes) and 11 hours (total load of 480 dynes) above the endogenous contraction force.
The effect of changing shape of the FPCL from long/thin to short/wide was investigated by re-orientating the lattice from a Low Aspect Ratio (LAR) lattice configuration (ratio of length to width), with attachment bars on the long edges of the lattice, to a High Aspect Ratio lattice configuration (HAR), with attachment bars placed on the shorter edges (FIG 3.7.2). Unless otherwise stated all FPCLs were cast as LAR lattices.
FIGURE 3.7.1. - Schematic diagram of Continuous linear loading regimes—

(A) Unloading and Loading for 11 hours (B), 1 hour (C) and 10 mins (D).

KEY — — projected cellular response

— mechanical loading

**Figure A**
- Endogenous contraction force

**Figure B**
- Endogenous contraction force
FORCE (dynes)

Endogenous contraction force

TIME (hours)

FORCE (dynes)

Endogenous contraction force

TIME (hours)
FIGURE 3.7.2.- Schematic diagram of (A) Low Aspect Ratio lattice (LAR) and (B) High Aspect Ratio lattice (HAR).

A

- Flotation bar
- Wire frame
- Petri dish
- Fibroblast populated collagen lattice

B

- Flotation bar
- Wire frame
- Petri dish
- Fibroblast populated collagen lattice
CHAPTER 4 - RESULTS

The ability to culture fibroblasts within a 3-D fibrillar collagen lattice provides an experimental model to monitor the response of cells to endogenous and exogenous mechanical forces applied upon it.

During this study, collagen lattice contraction was compared with untethered (non-load) FPCL, which has been used widely as a standard \textit{in vitro} model for contraction (Grinnell, 1994). Analysis of culture media conditioned from (1) non-loaded (untethered), (2) static loaded (tethered endogenously contracted FPCLs), and (3) externally loaded FPCLs, demonstrated the presence of a range of metallo- and serine proteases and TIMP in latent and active forms by zymography, Western blot analysis and immunostaining of FPCLs.

All enzyme activities were means of 3 separate FPCL experiments from each dermal specimen. Fibroblasts from duplicate specimens (age and site matched) were used. Fibroblasts from both specimens showed similar trends in their protease profiles to mechanical stimulation. However, differences in the actual levels of enzyme released were found apparently due to specimen (patient) variability.
4.1. - STATIC AND CYCLICAL LOADING

4.1.1.- Zymography Analysis

4.1.1.1.- Enzyme Release over 48 hours

Total MMP-2 and MMP-9 release rates were compared at the 8, 24 and 48 hour stages of endogenous static load (tethered lattice) by zymography (FIG 4.1.1). Greatest rate of release of MMP activity was observed at 8 hours, decreasing over 24 and 48 hours for both MMPs. At each time, total activity of MMP-2 was in excess of MMP-9. MMP-2 release rates were 2.8, 2.5 and 12.8 fold higher than MMP-9, at 8, 24 and 48 hours respectively. All subsequent experiments were terminated at 24 hours on completion of loading regimes.

FIGURE 4.1.1.- Rate of MMP-2 and MMP-9 release over 8, 24, and 48 hours during static loading. Total rate of enzyme release for both enzymes decreased with over 48 hours. (n=3)
Results in this section have been qualitatively examined in 4.1.1.2, and quantitatively in 4.1.1.3 and 4.1.1.4.

4.1.1.2.- Gelatin and Casein Zymograms

Figures 4.1.2 and 4.1.3 illustrate the enzyme activities of MMP-2 and MMP-9 on gelatin, and MMP-3 on casein zymograms, using 24 hour conditioned media collected from non-loaded (untethered), static loaded (tethered), and cyclically loaded FPCLs containing dermal fibroblasts from a 45 year old patient.

MMP-2 and MMP-9 activity increased with external mechanical tension during cyclical loading (FIG 4.1.2.). The bands for latent and active MMP-2 at 72kDa and 66kDa were shown to have increased activity with Cyclical Over Load (COL) (lane C) and Cyclical Under Load (CUL) (lane D), compared to non-loaded (lane A) and static loaded lattices (lane B).

MMP-9 active enzyme bands at 80kDa and 84kDa showed a marked increase in protease activity following static loading (lane B), COL (lane C) and CUL (lane D) relative to non-loaded lattices (lane A). The latent MMP-9 band at 92kDa was present in all four conditioned media samples and did not appear to change with loading.
FIGURE 4.1.2. (A) Gelatin zymogram- Enzyme activities of MMP-2 and MMP-9 following loading. (B) Scan of gelatin zymogram showing intensities of enzyme bands.

**Key-**

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>MMP-2 Standard</td>
</tr>
<tr>
<td>B</td>
<td>Conditioned medium from <em>untethered</em> FPCL</td>
</tr>
<tr>
<td>C</td>
<td>Conditioned medium from <em>static loaded</em> FPCL</td>
</tr>
<tr>
<td>D</td>
<td>Conditioned medium from <em>COL</em> FPCL</td>
</tr>
<tr>
<td>E</td>
<td>Conditioned medium from <em>CUL</em> FPCL</td>
</tr>
<tr>
<td>F</td>
<td>MMP-9</td>
</tr>
<tr>
<td>G</td>
<td>Baseline</td>
</tr>
</tbody>
</table>

**SCAN only**

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>72kDa Latent MMP-2</td>
</tr>
<tr>
<td>II</td>
<td>66kDa Active MMP-2</td>
</tr>
<tr>
<td>III</td>
<td>45kDa Active MMP-2</td>
</tr>
<tr>
<td>IV</td>
<td>92kDa Latent MMP-9</td>
</tr>
<tr>
<td>V</td>
<td>84kDa Active MMP-9</td>
</tr>
<tr>
<td>VI</td>
<td>80kDa Active MMP-9</td>
</tr>
</tbody>
</table>
The casein zymogram demonstrated that the MMP-3 activity decreased with external mechanical tension (FIG 4.1.3.). Conditioned medium from non-loaded, static loaded, COL, and CUL lattices did not show the latent MMP-3 activity at 55kDa. However, active MMP-3 bands at 45kDa and 28 kDa were observed with non-loaded (lane A) and static loaded lattices (lane B), but, only at 45kDa with COL (lane C) and CUL (lane D) conditioned media. Non-enzymatic protein bands present within the samples were detected as dark bands on the zymogram.
FIGURE 4.1.3.- (A) Casein zymogram- Enzyme activities of MMP-3 following loading. (B) Scan of casein zymogram showing intensities of enzyme bands.

Key- ZYMOGRAM and SCAN SCAN only
Lane A- MMP-3 Standard 1-57kDa Latent MMP-3
Lane B- Molecular weight standard II-45kDa Active MMP-3
Lane C- Conditioned medium from untethered FPCL III-28kDa Active MMP-2
Lane D- Conditioned medium from static loaded FPCL
Lane E- Conditioned medium from COL FPCL
Lane F- Conditioned medium from CUL FPCL
Lane G- Baseline

124
4.1.1.3. Non-loaded (nil-tension) vs. Static load (tethered FPCL).

At 24 hours, the total MMP-2 release rate was slightly reduced during static load relative to the non-loaded lattice (FIG 4.1.4.). However, total MMP-9 was significantly increased during static load by 4 fold, with the increase being mainly with the active form (FIG 4.1.5.). Total MMP-3 was only increased slightly during tethered lattice contraction by 1.6 fold (FIG 4.1.6).

Plasminogen activator release rates were also compared during non-load and static loads. In this case, the total enzyme released was increased with uPA and tPA activity rising by 6 and 7 fold respectively, following static load (FIG 4.1.7.).

4.1.1.4. Non-loaded (nil-tension) vs. Cyclical loads (COL and CUL).

At 24 hours, total MMP-2 release rates were significantly increased following cyclical loading in comparison with non-loaded FPCLs. Total enzyme activity rose by 2 and 1.6 fold for COL and CUL respectively (FIG 4.1.4.). MMP-2 was detected mostly in the latent form, but, both latent and active forms of MMP-2 were shown to increase with cyclical loading. Latent MMP-2 increased only by 1.5 and 1.3 fold, with COL and CUL, respectively, however, a greater increase was observed with active MMP-2 which rose by 4.8 and 3.3 fold, respectively.

Total MMP-9 release rates were demonstrated to be significantly higher than non-loaded lattices following cyclical loading. With an increase of 18 and 37 fold during
COL and CUL, respectively (FIG 4.1.5). Most of MMP-9 was detected in the active form, but, both active and latent forms increased with cyclical loading. Active MMP-9 rose by 21 and 38 fold, during COL and CUL, respectively, whereas, the latent form rose by 6 and 29 fold, respectively.

The active form of MMP-3 was only detected during cyclical loading. It was shown to significantly decrease to 40% and 56% of activity present in non-loaded lattices during COL and CUL, respectively (FIG 4.1.6.).

uPA release rates were found to be below detection limits during COL and CUL. Total tPA production increased significantly by 55 and 22 fold during COL and CUL, respectively, in comparison with non-loaded lattices (FIG 4.1.7.).

The contraction forces generated by fibroblasts during COL and CUL were measured and recorded by the t-CFM (FIG 4.1.8). Cellular activity was between 50-70 dynes at 8 hours when cyclical loading commenced either Over or Under endogenous contraction force.
** FIGURE 4.1.4. - Comparison of MMP-2 release in medium conditioned for 24 hours during Non-load (FF gel), Static loading (Endogenous contraction), and Cyclical loading (Cyclical Over Load and Cyclical Under Load) of FPCLs.

** p ≤ 0.01 COL and CUL vs. non-loaded FPCL (n=3)
FIGURE 4.1.5.- Comparison of MMP-9 release in medium conditioned for 24 hours during Non-load (FF gel), Static loading (Endogenous contraction), and Cyclical loading (Cyclical Over Load and Cyclical Under Load) of FPCLs.

*** p < 0.001 Static load, COL, and CUL vs. non-loaded FPCL (n=3)
FIGURE 4.1.6.- Comparison of MMP-3 release in medium conditioned for 24 hours during Non-load (FF gel), Static loading (Endogenous contraction), and Cyclical loading (Cyclical Over Load and Cyclical Under Load) of FPCLs.

** p ≤ 0.01 Static load, COL, and CUL vs non-loaded FPCL (n=3)
FIGURE 4.1.7.- Comparison of uPA (A) and tPA (B) release in medium conditioned for 24 hours during Non-load (FF gel), Static loading (Endogenous contraction), and Cyclical loading (Cyclical Over Load and Cyclical Under Load) of FPCLs.

(A) * p < 0.05 Static load vs. non-loaded FPCL

(B) * p < 0.05 CUL vs. non-loaded FPCL, ** p < 0.01 COL vs non-loaded FPCL

(n=3)
FIGURE 4.1.8.- (A) Cyclical Over Load and (B) Cyclical Under Load of FPCLs.

The loading cycles commenced at 8 hours, on completion of the main contraction period, each cycle was for 1 hour, and was repeated 16 times over a further 16 hour period.
4.1.2. - Analysis by Western Blotting

A single sample of conditioned medium from non-load, static load and cyclical loaded FPCL was analysed by Western blotting. Proteases detected by this method were mostly present in their latent forms (FIG 4.1.9.).

MMP-1 was observed mainly in the latent form at 55kDa, with the amount released appearing to increase during static and cyclical loaded lattices. Also in media collected during CUL the active form at 45 kDa was detected.

MMP-2 and MMP-9 were present only in their latent forms at 72kDa and 92kDa, respectively. From these blots it appears as if these enzymes were approximately equal with static and cyclical loading. The non-loaded lattice sample appeared unexpectedly to contain more enzyme than those samples from static and cyclically loaded lattices, possibly due to the presence of inactive antigen.

MMP-3 was seen to be released into the media during loading of all lattices. It was mainly observed in its latent form at 55kDa but for the non-loaded and static loaded lattices, which appeared to produce more MMP-3. With the active form at 45kDa present in these samples.
TIMP-1 was shown to be present in all loading regimes. Its release appeared to decrease with static and cyclically loaded samples, with the greatest reduction during CUL.

FIGURE 4.1.9.- Western blot analysis of Non-load, Static load and Cyclically loaded conditioned media samples.

**Key:**
- CL - MMP-1
- GA - MMP-2
- ST - MMP-3
- GB - MMP-9
- TIMP-1

Lane A- Conditioned medium from non-loaded FPCL

Lane B- Conditioned medium from static loaded FPCL

Lane C- Conditioned medium from COL FPCL

Lane D- Conditioned medium from CUL FPCL
4.1.3. - Immunolocalisation of MMPs and TIMP

Immunolocalisation of MMP-1, MMP-2, MMP-3, MMP-9, and TIMP-1 within the FPCLs following non-load, static load and cyclical loading demonstrated the presence of intracellular enzyme and inhibitor apparently within the Golgi apparatus of the cells. The enzyme accumulated within the cells and was not enhanced by including the secretory blocking agent monensin in the media, but was due to natural vesicular accumulation following the 3 loading regimes.

The strongest staining appeared in all lattices for MMP-1 followed by MMP-3 and TIMP-1 (FIG 4.1.10). The greatest enzyme accumulation occurred within cyclically loaded lattices showing widespread distribution throughout the cytoplasm. No cellular alignment was evident in any of the lattices, though, fibroblasts in non-loaded lattices were mostly rounded with a few short processes. Within static and cyclically loaded lattices fibroblasts were mostly bipolar with longer processes.
FIGURE 4.1.10. - Immunostaining of Non-load, Static load and Cyclically loaded FPCLs.

(A) **Non-loaded lattice**
Positive staining for MMP-3, stained with anti-serum to MMP-3 and detected by a FITC labeled secondary antibody, was present in granules around the nucleus, consistent with accumulations in the Golgi apparatus and secretory vesicles. The fibroblast was stellate with a few short processes following lattice contraction.

(Bar = 20μm)

(B) **Static loaded lattice**
Following static loading fibroblasts were bipolar and positive staining for MMP-1 (stained with anti-serum to MMP-1) was increased within the cell body.

(Bar = 20μm)

(C and D) **Cyclical loaded lattice**
Following cyclical loading fibroblasts were bipolar with longer cell processes. Positive staining for MMP-1 (stained with anti-serum to MMP-1) was observed throughout the cell body (C) and was present in the cell process (D).

(Bar = 20μm)
4.1.4. - SUMMARY OF RESPONSES TO STATIC AND CYCLICAL LOADING REGIMES.

A comparison of non-loaded (nil-tension), static load (tethered endogenous contraction), and cyclical loaded (exogenous cyclical loading) FPCLs demonstrated a range of metallo- and serine protease profiles present in the conditioned medium. These profiles indicated substantial and complexed sensitivities of the production and distribution of proteases to different mechanical regimes. Overall results from all 3 methods of enzyme detection are summarized in Table 4.1.1.

Table 4.1.1. - Summary of protease and inhibitor response to static and cyclical loads.

The arrows indicate the rise or fall of the protease or inhibitor in relation with the non-loaded lattice (↔).

<table>
<thead>
<tr>
<th>PROTEASE/INHIBITOR</th>
<th>NON-LOAD</th>
<th>STATIC LOAD</th>
<th>CYCLICAL OVER LOAD</th>
<th>CYCLICAL UNDER LOAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>↔</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>MMP-2</td>
<td>↔</td>
<td>↑</td>
<td>↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>MMP-3</td>
<td>↔</td>
<td>↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>MMP-9</td>
<td>↔</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>uPA</td>
<td>↔</td>
<td>↑↑</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>tPA</td>
<td>↔</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>↔</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
</tbody>
</table>

KEY  
↑↓ slight increase/decrease  
↑↑ moderate increase  
↑↑↑ substantial increase  
* "denotes detection was below the limits of the technique used
(A) MMP-1 increased with cyclical loading.

(B) MMP-2 was largely insensitive to loading, with similar rates of enzyme release apparent in loaded and unloaded lattices.

(C) MMP-9 was highly responsive to mechanical loading, showing dramatic increases following cyclical loading.

(D) MMP-3 and TIMP-1 showed a modest decrease in production with loading.

(E) uPA increased with static load, but was reduced following cyclical loading.

(F) tPA increased substantially with cyclical loading.

(G) Distribution of MMPs and inhibitor increased throughout the cell body and into the cell processes with tethered and cyclical loading. No cellular alignment was detected within the Low Aspect Ratio lattices.
4.2. - EFFECTS OF SOURCE-TISSUE AGE TO STATIC AND CYCLICAL LOADING REGIMES.

The previous section has described how protease profiles were changed with exogenous mechanical loading. To establish whether these trends were reproducible for specimens taken from patients of different ages three age groups were selected with their mean age at 15, 30, 45, additionally a 60 year old specimen was also included.

Duplicate age and site matched dermal specimens were used for each group. All experiments were performed using fibroblasts at passage 4. The protease responses from these groups were examined following non-load, static, and Cyclical Over Loading. All enzyme activities were means of 3 separate FPCL experiments.
4.2.1. Endogenous contraction of fibroblasts from increasing source-tissue age.

Endogenous contraction forces generated by fibroblasts from all groups were shown to be responsive to static load (FIG 4.2.1). All measurements of endogenous contraction were means of triplicate experiments. The greatest contraction was produced by 15 year old group. While the 30 and 45 year old groups had similar contractility, but surprisingly, contraction was much higher in the 60 year old specimen in contrast to the 30 and 45 year old groups.

FIGURE 4.2.1.- Endogenous contraction of fibroblasts with increasing source-tissue age. (n=3)
A similar trend was observed when the initial rate of contraction was monitored over the first 3 hours of contraction (FIG 4.2.2). For all age groups the greatest rate of contraction occurred within the first 10 mins, this was followed by a sharp decrease in the contraction rate for all groups. The 15 year old group maintained the highest rate of contraction throughout the 3 hour period. With the rate decreasing during most of the period, with the exception of a few small rises. For the 30 and 45 year age groups, there was a rapid fall in contraction after the first 10 mins, with the rate staying more or less constant with a few fluctuations throughout 3 hours. The 60 year old specimen showed the greatest variation in the rate of contraction over 3 hours. The rate was below the 15 year old group and by 3 hours it was steady, with a similar rate to the 30 and 45 year old groups.

FIGURE 4.2.2.- Initial rate of contraction of fibroblasts from all age groups.
Table 4.2.1. summarizes the maximum rate of contraction, endogenous contraction force generated at 10 hours, and maximum force generated at 24 hours by all age groups. These results show that in all cases the highest forces generated were with the 15 year group. Comparable rates of contraction were measured with the 30 and 45 year age groups. However, the endogenous contraction force at 10 hours and the maximum force monitored at 24 hours were substantially higher in the 60 year old specimen.

Table 4.2.1- Forces generated by fibroblasts from different ages of source-tissue.

<table>
<thead>
<tr>
<th>MEAN AGE GROUP (years)</th>
<th>MAXIMUM RATE OF CONTRACTION (dynes/ hour)</th>
<th>ENDOGENOUS FORCE AT 10 HOURS (dynes)</th>
<th>MAXIMUM FORCE GENERATED AT 24 HOURS (dynes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>86.2 (±8.4)</td>
<td>180.1 (±43.3)</td>
<td>228.2 (±34.2)</td>
</tr>
<tr>
<td>30</td>
<td>63.0 (±6.2)</td>
<td>42.2 (±5.1)</td>
<td>64.4 (±8.0)</td>
</tr>
<tr>
<td>45</td>
<td>71.9 (±4.3)</td>
<td>58.8 (±4.5)</td>
<td>86.4 (±7.4)</td>
</tr>
<tr>
<td>60</td>
<td>54.1 (±7.0)</td>
<td>108.1 (±12.5)</td>
<td>204.2 (±13.5)</td>
</tr>
</tbody>
</table>
4.2.2.- Production of MMP-2 and MMP-9 with increasing age of source-tissue.

Fibroblasts from each age group (15, 30, 45, and 60 year old specimen) had similar trends in their protease profiles for MMP-2 and MMP-9 following non-load, static load and COL.

For 15 and 45 year old age groups total MMP-2 release rate increased significantly with COL compared to non-loaded lattices (FIG 4.2.3). A rise of 2.3 and 2 fold were monitored with the 15 and 45 year old groups, respectively. However, there was no substantial difference in the 30 year old group and 60 year old specimen. Although the 30 year old group had consistently higher levels of MMP-2 than the other groups.

MMP-2 was mainly present in its latent form in all groups. Increases in the active forms were seen following loading of 15 and 45 year old group (FIG 4.2.4.). For the 15 year group the latent form increased by 1.4 and 2 fold following static loading and COL, respectively, and active MMP-2 rose by 3.6 fold following COL. Following static and COL, active MMP-2 for the 45 year old group rose by 1.8 and 4.8 fold, respectively, but its latent form rose by only 1.5 fold following COL. For the 30 year old group, latent MMP-2 had very similar values with those measured following untethered lattice contraction. Active MMP-2 for the 30 year old group rose by 2.1 and 2.7 fold with static and cyclical loading, respectively.
FIGURE 4.2.3. - Comparison of MMP-2 release in medium conditioned for 24 hours during Non-load (FF gel), Static loading (Endogenous contraction), and Cyclical over loading (COL) of FPCLs.

A- 15 year group, * p ≤ 0.05 COL vs. non-loaded FPCL.

C- 45 year group, ** p ≤ 0.01 COL vs. non-loaded FPCL. (n=3)
FIGURE 4.2.4.- Comparison of latent and active MMP-2 release in medium conditioned for 24 hours during Non-load (FF gel), Static loading (Endogenous contraction), and Cyclical over loading (COL) of FPCLs. 

(n=3)
Total MMP-9 enzyme release rates increased significantly with static and COL for all groups in comparison with untethered lattices (FIG 4.2.5.). The greatest increases were detected for the 15 and 45 year old groups. For the 15 year old group, a rise of 2.3 and 3.5 fold was measured following static and COL, respectively. A substantial increase was seen for the 45 year old group following loading, such that MMP-9 rose by 4 and 18 fold (static and COL).

For each age group, active and latent forms of MMP-9 were shown to increase following both types of loading, although the active form dominated (FIG 4.2.6.). The most dramatic increases in the active enzyme were detected with the 45 year old group. Active MMP-9 increased by 4.8 and 21 fold following static and cyclical loading. Furthermore, the latent MMP-9 rose by 2.2 and 5.6 fold. For the 15 year old group, a rise of 6 and 7 fold was measured for active MMP-9 following static and COL, respectively, and latent MMP-9 for this group increased by 2.3 (COL). For the 30 year old group, active MMP-9 was shown to increase by almost 2 fold following cyclical loading, and a rise of 2.2 fold was measured for latent MMP-9 following static load.
FIGURE 4.2.5.- Comparison of MMP-9 release in medium conditioned for 24 hours during Non-load (FF gel), Static loading (Endogenous contraction), and Cyclical overloading (COL) of FPCLs.

A-15 year group, * p ≤ 0.05 Static load vs. non-loaded FPCL, ** p ≤ 0.01 COL vs. non-loaded FPCL. B-30 year group, * p ≤ 0.05 COL vs. non-loaded FPCL. C-45 year group, *** p ≤ 0.001 Static load and COL vs. non-loaded FPCL. D-60 year group, * p ≤ 0.05 COL vs. non-loaded FPCL. (n=3)
FIGURE 4.2.6.- Comparison of latent and active MMP-9 release in medium conditioned for 24 hours during Non-load (FF gel), Static loading (Endogenous contraction), and Cyclical over loading (COL) of FPCLs. (n=3)
4.2.3. - SUMMARY OF RESPONSES OF INCREASING SOURCE-TISSUE AGE TO STATIC AND CYCLICAL LOADING REGIMES

Comparison of non-loaded (nil-tension), static loaded (tethered endogenous contraction), and cyclical loaded (Cyclical Over Load) FPCLs over the age groups found:

(A) Endogenous contraction forces generated by fibroblasts were shown to be responsive irrespective of patient age. The greatest contraction forces were present in the youngest group, and forces decreased for 30 and 45 year groups, but increased for the 60 year old specimen.

(B) All groups had similar protease profiles for MMP-2 and MMP-9 production irrespective of patient age. MMP-2 was not so sensitive to mechanical stimulation, whereas MMP-9 increased dramatically to cyclical loading. MMP-9 was mostly sensitive in the 15 year old group, but it was highly activated in the 30 and 45 year old groups.
4.3. - **STATIC vs. CONTINUOUS LINEAR LOADING**

In this section the protease responses following continuous linear loading regimes were compared with endogenous static loading (tethered FPCLs). Exogenous continuous loads were precisely applied using the t-CFM and were initiated at 8 hours of contraction (Section 3.7, FIG 3.7.1.). Loads were applied for 10 mins (total force of 4000 dynes), 1 hour (480 dynes) and 11 hours (480 dynes) above the endogenous contraction force generated by fibroblasts for continuous *loading* and for 11 hours during continuous *unloading* regimes (480 dynes).

Duplicate age and site matched dermal specimens were used, with fibroblasts from the 30 year old source-tissue age group. All experiments were performed using fibroblasts at passage 4 and 5. Protease responses were measured in medium following 24 hours of conditioning. All protease responses were means of triplicate FPCL experiments.
4.3.1.- Zymographic analysis

Total MMP-2 release rates for continuous linear loads were similar to endogenous static load (FIG 4.3.1). But, a significant change was measured following continuous loading for 11 hours, MMP-2 release rate was reduced by 30% of the endogenous static load.

MMP-2 was apparent in latent and active forms, in all cases the latent form was the predominant species (over 80%). Following continuous loading, the rate of release of the latent form was found to be similar to that detected following static loading, only a 30% decrease was measured following continuous loading at 11 hours compared to static loading. In contrast to latent MMP-2, active MMP-2 although minimal was found to increase by 3 fold following continuous loading and unloading.
FIGURE 4.3.1.-Comparison of MMP-2 release in medium conditioned for 24 hours during Endogenous static load (Endogenous contraction), Continuous loading for 10 mins, 1 hour, 11 hours, and Continuous unloading (11 hours Unload) of FPCLs.

** p ≤ 0.01 11 hours Continuous loading vs. Endogenous static load. (n=3)
Total MMP-9 release rates were demonstrated to increase significantly following all continuous loading and unloading regimes in comparison to static loading (FIG 4.3.2.).

Following the continuous loading regimes rises of 1.7, 2, and 5 fold were measured for 10 mins, 1 hour and 11 hours load, respectively. A rise of 5 fold was also measured following 11 hours unloading.

Latent and active forms of MMP-9 were detected in all loading regimes. In contrast to MMP-2, the active form of MMP-9 was the predominant species (over 90%), i.e. MMP-9 was fully activated. In all cases active MMP-9 was increased by 1.7 fold (10 mins load), 2.1 fold (1 hour load), and 5.3 fold (11 hours loading and unloading).
**FIGURE 4.3.2.-** Comparison of MMP-9 release in medium conditioned for 24 hours during Endogenous static load (Endogenous contraction), Continuous loading for 10 mins, 1 hour, 11 hours, and Continuous unloading (11 hours Unload) of FPCLs.

* $p \leq 0.05$ 10 mins and 1 hour Continuous loading vs. Endogenous static load.

*** $p \leq 0.001$ 11 hours Continuous loading and unloading vs. Endogenous static load.

(n=3)
Total MMP-3 release rates for continuous loading and unloading were all decreased relative to endogenous static loading (FIG 4.3.3). MMP-3 release rates were measured only at 7% and 8% following continuous loading for 10 mins and 1 hour, respectively. However, following 11 hours loading and unloading release rates were below detection limits.

![Graph showing MMP-3 release rates](image)

**FIGURE 4.3.3.-** Comparison of MMP-3 release in medium conditioned for 24 hours during Endogenous static load (Endogenous contraction), Continuous loading for 10 mins, 1 hour, 11 hours, and Continuous unloading (11 hours Unload) of FPCLs.

**p ≤ 0.01 10 mins and 1 hour Continuous loading vs. Endogenous static load. (n=3)**
Total uPA release rate was reduced significantly with all continuous loading and unloading regimes compared to endogenous static loading (FIG 4.3.4.). Following continuous loading enzyme activity had decreased to 30% (10 mins load) 40% (1 hour load) and 60% (11 hours load), and with continuous unloading (11 hours) the rate was lowered to 50%.

However, with total tPA, a significant increase in the rate of release was monitored following all continuous loading and unloading regimes (FIG 4.3.4). Increases of 1.4 fold (10 mins load), 1.8 fold (1 hour load) and 2 fold (11 hours load) were measured following continuous loading. With continuous unloading (11 hours) a rise of 1.4 fold was detected.
FIGURE 4.3.4.-Comparison of uPA (A) and tPA (B) release in medium conditioned for 24 hours during Endogenous static load (Endogenous contraction). Continuous loading for 10 mins, 1 hour, 11 hours, and Continuous unloading (11 hours Unload) of FPCLs.

(A) **p ≤ 0.01 10 mins, 1 hour, and 11 hours Continuous Load and 11 hours Unload vs. Endogenous static load.

(B) *p ≤ 0.05 10 mins Continuous load and 11 hours Continuous unload vs. Endogenous static load.

**p ≤ 0.01 1 hour and 11 hours Continuous load vs. Endogenous static load (n=3)
4.3.2. - Western blot analysis.

A single sample from each continuous loading and unloading FPCL conditioned medium was analysed by Western blotting. Fig 4.3.5. illustrates the proteases detected by this method were mostly present in their latent form.

MMP-1 was observed to increase modestly following continuous loading and unloading. Both latent (55kDa) and active (45kDa) forms of MMP-1 were apparent.

MMP-2 in all cases was observed to be similar to static loading. In contrast, MMP-9 was observed to increase with continuous loading and unloading. The active form (84 kDa) in addition to the latent form (92kDa) could be observed mostly with 11 hours continuous loading and unloading.

MMP-3 was apparent following all continuous loading regimes, but was not observed with continuous unloading.

TIMP-1 activity was detected following endogenous static load and a very faint band with 10 mins continuous loading. Bands were not apparent with 1 and 11 hours loading and 11 hours unloading.
FIGURE 4.3.5.- Western blot analysis of endogenous static and exogenous continuous linear loaded conditioned media samples.

Key:
- CL - MMP-1
- GA - MMP-2
- ST - MMP-3
- GB - MMP-9
- TIMP-1

Lane A- Conditioned medium from endogenously loaded FPCL
Lane B- Conditioned medium from continuously loaded 10 mins FPCL
Lane C- Conditioned medium from continuously loaded 1 hour FPCL
Lane D- Conditioned medium from continuously loaded 11 hours FPCL
Lane E- Conditioned medium from continuously unloaded 11 hours FPCL
4.4.3 - SUMMARY OF RESPONSES TO STATIC vs CONTINUOUS LINEAR LOADS.

Comparison of proteases following exogenous continuous linear loading and endogenous static loading regimes demonstrated that release rates were dramatically affected by exogenous loading. Overall results from both methods are summarized in table 4.3.1.

Table 4.3.1. - Summary of protease and inhibitor response to static vs. continuous linear loads. The arrows indicate the rise or fall of the protease or inhibitor in relation to endogenous static loading (↔).

<table>
<thead>
<tr>
<th>PROTEASE/INHIBITOR</th>
<th>END. LOAD</th>
<th>LOAD 10 MINS</th>
<th>LOAD 1 HOUR</th>
<th>LOAD 11 HOURS</th>
<th>UNLOAD 11 HOURS</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>↔</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>MMP-2</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>MMP-3</td>
<td>↔</td>
<td>↓</td>
<td>↓</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>MMP-9</td>
<td>↔</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>uPA</td>
<td>↔</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>tPA</td>
<td>↔</td>
<td>↑↑</td>
<td>↑↑↑</td>
<td>↑↑↑</td>
<td>↑</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>↔</td>
<td>↓</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>

KEY

↑/↓ slight increase/decrease

↑↑ moderate increase

↑↑↑ substantial increase

"*" denotes detection was below the limits of the technique used

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(A) The release rates of MMP-9 and tPA were found to be substantially up regulated following continuous loading and unloading, and modest increases were detected for MMP-1.

(B) MMP-2 was observed to be largely insensitive to continuous loading and unloading.

(C) MMP-3, TIMP-1 and uPA rates of release were decreased dramatically following continuous loading and unloading.

(D) Changes in protease synthesis appear to be initiated by the total length of the change (i.e. time the cells are under a changing load/unload), but not the change in the rate of strain (i.e. total force) applied to FPCL.
4.4. - **LOW ASPECT RATIO vs HIGH ASPECT RATIO LATTICE CONFIGURATION.**

In this section protease responses following High Aspect Ratio (HAR) lattice configuration were compared with those of Low Aspect Ratio (LAR) lattices. The aspect ratio for the HAR lattice (ratio of lattice length to lattice width, i.e. free edge to attachment bar) was 3:1, and 0.33:1 for the LAR lattice (Eastwood *et al.*, 1998). Fig 4.4.1. illustrates LAR and HAR lattice configuration on completion of the loading regimes at 24 hours. Both lattice configurations were indented in the central region (i.e. free edge) with the greatest change observed in the HAR lattice configuration.

Duplicate age and site matched dermal specimens were used, with fibroblasts from the 30 year old source-tissue age group. All experiments were performed using fibroblasts at passage 4 and 5. Protease responses were measured in medium following 24 hours of conditioning. All protease responses were means of triplicate FPCL experiments.
FIGURE 4.4.1 - (A) Low Aspect Ratio lattice at 24 hours and (B) High Aspect Ratio lattice configurations at 0 and 24 hours. (A- courtesy of K. Sethi, 1998)
4.4.1.- Zymographic analysis

Total MMP-2 release rate measured for both LAR and HAR lattices were found to be very similar following endogenous static loading (FIG 4.4.2.). There was a significant increase (1.4 fold) following HAR cyclical over loading compared to LAR cyclical over loading.

For both lattice configurations latent and active forms of MMP-2 were detected, although the latent MMP-2 was the dominant form (over 90%). A rise in latent form (by 1.4 fold) was measured following HAR cyclical over loading in comparison to LAR cyclical over loading. Following HAR endogenous static loading, the rate of release of latent MMP-2 remained constant, but active MMP-2 decreased its rate of release to 30% of that observed following LAR lattice static load.
FIGURE 4.4.2.- Comparison of MMP-2 release in medium conditioned for 24 hours during LAR Endogenous static loading (LAR Endogenous contraction), HAR Endogenous static loading (HAR Endogenous contraction), LAR Cyclical Over Loading (LAR COL), and HAR Cyclical Over Loading (HAR COL) of FPCLs.

*p ≤ 0.05 High Aspect Ratio Cyclical Over Load vs. Low Aspect Ratio Cyclical Over Load.

(n=3)
Total MMP-9 release rate was significantly increased following HAR endogenous static loading and cyclical over loading compared to LAR loaded lattices (FIG 4.4.3.). A rise of 1.7 fold and 2.6 fold was detected following HAR endogenous static load and cyclical over load, respectively.

In addition, these increases were mirrored with the rate of release of active MMP-9, the dominant enzyme form (over 90%). Rises of 1.8 and 2.6 fold were measured following HAR endogenous static load and cyclical over load in comparison with LAR loaded lattices. Latent MMP-9 release was also affected by the HAR lattice configuration. It’s release was reduced to 76% of that following endogenous static load, and was increased by 3.3 fold following cyclical over load compared to LAR lattices.
FIGURE 4.4.3.- Comparison of MMP-9 release in medium conditioned for 24 hours during LAR Endogenous static loading (LAR Endogenous contraction), HAR Endogenous static loading (HAR Endogenous contraction), LAR Cyclical Over Loading (LAR COL), and HAR Cyclical Over Loading (HAR COL) of FPCLs.

*p ≤ 0.05 High Aspect Ratio Endogenous static load vs. Low Aspect Ratio Endogenous static load.

***p ≤ 0.001 High Aspect Ratio Cyclical Over Load vs. Low Aspect Ratio Cyclical Over Load.

(n=3)
Total MMP-3 release rate was demonstrated to be significantly reduced following both HAR endogenous static loading and HAR cyclical over loading in comparison to LAR loaded lattices (FIG 4.4.4.). Following HAR endogenous static load MMP-3 was reduced by 40% of the LAR lattice release rate, whilst a reduction of 80% followed HAR cyclical over load.

**FIGURE 4.4.4.-** Comparison of MMP-3 release in medium conditioned for 24 hours during LAR Endogenous static loading (LAR Endogenous contraction), HAR Endogenous static loading (HAR Endogenous contraction), LAR Cyclical Over Loading (LAR COL), and HAR Cyclical Over Loading (HAR COL) of FPCLs.

**p ≤ 0.01** High Aspect Ratio Endogenous static load vs. Low Aspect Ratio Endogenous static load.

**p ≤ 0.001** High Aspect Ratio Cyclical Over Load vs. Low Aspect Ratio Cyclical Over Load. (n=3)
As MMP-3, total uPA release rate, was significantly decreased following HAR endogenous static loading and cyclical over loading in comparison to LAR lattices (FIG 4.4.5.). Enzyme production was reduced by 85% and 75% of LAR loaded lattices following HAR endogenous static loading and HAR cyclical over loading, respectively.

Unlike uPA, total tPA release rate was significantly increased with both HAR loading regimes. It rose by 1.5 fold and 1.8 fold following HAR endogenous static loading and HAR cyclical over loading, respectively, compared to LAR lattices.
FIGURE 4.4.5.-Comparison of (A) uPA and (B) tPA release rate in conditioned medium following LAR Endogenous static load (Endogenous contraction), HAR Endogenous static load (HAR Endogenous contraction), LAR Cyclical Over Load (COL), and HAR Cyclical Over Load (HAR COL) loading regimes at 24 hours.

(A) **p < 0.01 High Aspect Ratio Endogenous static load and Cyclical Over Load vs. Low Aspect Ratio Endogenous static load and Cyclical Over Load.

(B) *p < 0.05 High Aspect Ratio Endogenous static load vs. Low Aspect Ratio Endogenous static load.

**p < 0.01 High Aspect Ratio Cyclical Over Load vs. Low Aspect Ratio Cyclical Over Load. (n=3)
4.4.2. Western blot analysis.

A single sample from each of LAR and HAR endogenous static loaded and cyclical over loaded conditioned medium was analysed by Western blotting. Fig 4.4.6. illustrates proteases detected by this method were mostly all present in their latent forms.

MMP-1 was observed to increase dramatically following loading with both HAR lattice configurations.

MMP-2 was observed to increase slightly with both HAR lattice configurations compared to the LAR lattices. However, greater increases in MMP-9 were detected following both HAR lattice loadings compared to LAR lattices.

MMP-3 was produced following loading with both types of lattices (LAR and HAR), but bands were much fainter in HAR lattices.

TIMP-1 activity appeared to increase with loading and HAR lattice configuration.
FIGURE 4.4.6.- Western blot analysis of Low Aspect Ratio and High Aspect Ratio loaded conditioned media samples.

**Key:**
- CL - MMP-1
- GA - MMP-2
- ST - MMP-3
- GB - MMP-9
- TIMP-1

Lane A-Conditioned medium from *Low Aspect Ratio endogenous static loaded* FPCL

Lane B-Conditioned medium from *High Aspect Ratio endogenous static loaded* FPCL

Lane C-Conditioned medium from *Low Aspect Ratio cyclical over load* FPCL

Lane D-Conditioned medium from *High Aspect Ratio cyclical over load* FPCL
4.4.3. - Immunolocalisation of MMPs and TIMP

Analysis of LAR and HAR static loaded and cyclical over loaded FPCLs for MMP-1, MMP-2, MMP-3, MMP-9, and TIMP-1 by immunolocalisation demonstrated the production of all the enzymes and inhibitor by all lattices. More positive cells with enzyme and TIMP-1 were detected in lattices following HAR static load and HAR cyclical over load regimes.

Within HAR lattices, fibroblasts appeared to be aligned within certain regions of the lattice, these were the regions subjected to significant uniaxial lines of strain- zones 1 and 3 (FIG 4.3.1.). Strain lines were predicted by computer analysis (Eastwood et al., 1998). These fibroblasts appeared to be bipolar with long processes. Intense staining for MMPs and TIMP was concentrated not only around the golgi apparatus, but spread throughout the cell body and processes. However, cells in regions of the lattice which were less subjected to uniaxial lines of strain (zone 2) were found to be non-aligned and stellate in appearance. Within these cells, enzymes and inhibitor were localised solely around the golgi apparatus.

Examination of LAR static loaded and LAR cyclical over loaded lattices demonstrated that the enzyme and inhibitor appeared to increase with cyclical loading, even though these cells were all non-aligned (Section 4.1.3.).
FIGURE 4.4.7. Immunostaining of HAR lattices following endogenous static loading and cyclical over loading.

A - Schematic diagram of HAR lattice following loading regimes. Numbers represent regions investigated within the lattice.

Zone 1 and 3

Fibroblasts within these zones were found to be mostly bipolar and aligned. (B) and (C) illustrate bipolar fibroblasts from zone 1 following static and cyclical loading, respectively, stained with anti-serum to MMP-1 and detected by a FITC labeled secondary antibody. Strong positive staining for MMP-1 was found within these fibroblasts, both around the Golgi apparatus and widespread throughout the main cell body and within the cell processes. (D) illustrates bipolar fibroblasts from zone 3 following cyclical loading stained with anti-serum to MMP-3. Within these cells positive MMP-3 staining was located predominantly to one side of the cell (arrows). This side of the cell was nearest the lattice edge and was highly affected by cyclical loading. (Bar = 20μm)
Zone 2

(F) Fibroblasts within this region stained with antisera to MMP-1 were least affected by static and cyclical loading. Cells were mostly stellate and non-aligned and positive MMP-1 staining was present around the nuclei. (Bar = 20µm)
4.4.4 - SUMMARY OF RESPONSES TO LOW ASPECT RATIO vs. HIGH ASPECT RATIO LATTICE CONFIGURATION.

Comparison of proteases following LAR and HAR static and cyclical loading regimes has demonstrated that a change in lattice configuration could affect and alter protease production. Overall results from the three methods are summarized in table 4.4.1.

Table 4.4.1 - Summary of protease and inhibitor response to LAR and HAR endogenous static load and cyclical over load. The arrows indicate the rise or fall of the protease or inhibitor in relation to LAR endogenous static loading and LAR cyclical over load (↔).

<table>
<thead>
<tr>
<th>PROTEASE/INHIBITOR</th>
<th>LAR END. STATIC LOAD</th>
<th>HAR END. STATIC LOAD</th>
<th>LAR CYCLICAL OVER LOAD</th>
<th>HAR CYCLICAL OVER LOAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>↔</td>
<td>↑↑</td>
<td>↔</td>
<td>↑↑</td>
</tr>
<tr>
<td>MMP-2</td>
<td>↔</td>
<td>↑</td>
<td>↔</td>
<td>↑↑</td>
</tr>
<tr>
<td>MMP-3</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
<td>↓</td>
</tr>
<tr>
<td>MMP-9</td>
<td>↔</td>
<td>↑↑</td>
<td>↔</td>
<td>↑↑↑↑</td>
</tr>
<tr>
<td>uPA</td>
<td>↔</td>
<td>↓</td>
<td>↔</td>
<td>↓</td>
</tr>
<tr>
<td>tPA</td>
<td>↔</td>
<td>↑↑</td>
<td>↔</td>
<td>↑↑↑↑</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>↔</td>
<td>↑↑</td>
<td>↔</td>
<td>↑↑</td>
</tr>
</tbody>
</table>

KEY ↑/↓ slight increase/decrease, ↑↑ moderate increase, ↑↑↑↑ substantial increase
(A) Rates of release of MMP-1, MMP-9, TIMP-1, and tPA were found to be sufficiently up-regulated following loading regimes using the HAR lattice configuration.

(B) Release rate of MMP-2 was shown only to be responsive to HAR cyclical overloading.

(C) Production of MMP-3 and uPA were decreased following loading regimes using the HAR lattice configuration.

(D) The protease response following static or cyclical loading in LAR lattices was enhanced by HAR lattices, i.e. same but bigger or smaller.

(E) Fibroblasts were demonstrated to be aligned parallel to uniaxial lines of strain in HAR lattices, this was not observed in LAR lattices. This affected the localisation of MMPs and TIMP within the cell which were seen to follow the alignment.
4.5. - EFFECTS OF PATHOLOGICAL TISSUES TO STATIC AND CYCLICAL LOADING REGIMES.

The previous sections have dealt with endogenous and exogenous loading of normal fibroblasts, in this section the effect of protease regulation from keloid and scar fibroblasts has been examined following non-loaded, static loaded, and cyclical over loaded loading regimes.

4.5.1. - REGULATION OF PROTEASES FROM KEOID FIBROBLASTS

All keloid fibroblasts used in FPCLs were in the LAR lattice configuration. Details of the keloidal tissue used are shown in table 4.5.1.

Table 4.5.1- Data from keloid tissue

<table>
<thead>
<tr>
<th>PATIENT</th>
<th>GENDER</th>
<th>AGE</th>
<th>SITE OF KEOID</th>
<th>DURATION OF KEOID (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>Male</td>
<td>25</td>
<td>Neck</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>26</td>
<td>Left clavicle</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>27</td>
<td>Right shoulder</td>
<td>4</td>
</tr>
<tr>
<td>4</td>
<td>Male</td>
<td>32</td>
<td>Right cheek</td>
<td>3</td>
</tr>
<tr>
<td>5*</td>
<td>Male</td>
<td>35</td>
<td>Sternal area</td>
<td>10</td>
</tr>
</tbody>
</table>

denotes "*" previous steroid therapy

Mean age of patients 29 years.
4.5.1.1.- Endogenous contraction of keloid fibroblasts

Endogenous contraction forces generated by keloid fibroblasts (25-35 years, passage 4) were compared with normal fibroblasts (30 year old, passage 4) over 24 hours (FIG 4.5.1). Endogenous contraction at 8 hours for keloid fibroblasts was 184.4 ± 19.0 dynes, but for normal fibroblasts it was 46.5 ± 5.1 dynes, giving a percentage difference in 8 hour contraction of 75%. This difference in contraction was maintained throughout the experiment. By 24 hours, the maximum force generated by keloid fibroblasts was 287.6 ± 25.2 dynes compared to 64.4 ± 9.1 dynes generated by normal fibroblasts, a difference in force of 78%. At both time points the contraction forces generated by keloid fibroblasts were significantly increased than normal fibroblasts.

FIGURE 4.5.1.-Endogenous contraction forces generated from normal and keloid fibroblasts. ** p ≤ 0.01 keloid vs. normal fibroblasts at 8 and 24 hours. Dotted line represents contraction at 8 hours.
The initial rate of contraction over 10 hours was greatest for normal fibroblasts during the first 30 mins at 63 dynes/hour (FIG 4.5.2). This rate of contraction dramatically decreased by 1 hour, and remained relatively constant after 2 hours. A sharp rise in the rate of contraction for keloid fibroblasts was detected from 1 hour onwards. It steadily increased and peaked at 3.5 hours with a maximum rate of contraction of 53 dynes/hour. The keloid fibroblasts maintained a greater contraction rate from 1 to 7 hours, after which it gradually decreased. By 10 hours the rate of contraction for both cultures were getting closer, but the rate was always greater for keloid fibroblasts.

FIGURE 4.5.2.-Rate of contraction generated from normal and keloid fibroblasts, over first 10 hours of endogenous contraction.
Conditioned medium collected following non-loaded, endogenous static load and Cyclical Over Load (COL) FPCLs from ‘patient 4’ (in Table 4.5.1.) were used for zymography (MMP-2 and MMP-9 only) and Western blot analysis. All protease responses were means of triplicate FPCL experiments. Keloid fibroblasts at passage 4 were used.

4.5.1.2.- Zymographic Analysis

Total MMP-2 release rate was not significantly different following static loading, but was significantly reduced to 60% following COL in comparison to non-loaded lattices (FIG 4.5.3.). Latent MMP-2 was decreased by 35% following COL, leaving almost no (only 2%) MMP-2 in the active form following COL.
FIGURE 4.5.3. - Comparison of MMP-2 release rate in medium conditioned for 24 hours during Non-loaded (FF gel), Endogenous static loading (Endogenous contraction), Cyclical Over Loading (COL) of FPCLs.

** p ≤ 0.01 Cyclical Over Load vs. Non-loaded FPCL (n=3)
Total MMP-9 release increased significantly following both loading regimes in comparison to non-loaded lattices (FIG 4.5.4.). A rise of 3.2 fold and 4.5 fold were monitored following static load and COL, respectively.

MMP-9 was present mostly in the active form. The active MMP-9 increased by 4.3 fold (static load) and 5.6 fold (COL) compared to non-loaded FPCLs. Latent MMP-9 also rose following loading but only by 1.4 fold (static load) and 2.4 fold (COL).
FIGURE 4.5.4. - Comparison of MMP-9 release rate in medium conditioned for 24 hours during Non-loaded (FF gel), Endogenous static loading (Endogenous contraction), Cyclical Over Loading (COL) of FPCLs.

** p ≤ 0.01 Endogenous static load vs. Non-loaded FPCL

*** p ≤ 0.001 Cyclical Over Load vs. Non-loaded FPCL

(n=3)
4.5.1.3. Western blot Analysis

A single sample of conditioned medium from each of non-loaded, static loaded, cyclical over loaded FPCLs was analysed by Western blotting. Fig 4.5.5. illustrates proteases detected by this method were mostly present in their latent forms.

MMP-1 released into the media was observed to increase dramatically following static loading in comparison to non-loaded lattice. However, far less MMP-1 was present from COL. Both latent and active MMP-1 were seen for all three loading regimes.

There appeared to be less MMP-2 (latent form only) following static and COL in comparison to non-loaded lattice. In contrast MMP-9 increased with loading, especially with COL, both latent and active forms were observed.

MMP-3 was shown to decrease substantially following static and cyclical loading, with only the latent form present.

TIMP-1 activity remained constant between all loading regimes.
FIGURE 4.5.5.- Western blot analysis of Non-loaded, Static loaded and Cyclical Over Loaded conditioned media samples using keloid fibroblasts.

**Key-**
- CL - MMP-1
- GA - MMP-2
- ST - MMP-3
- GB - MMP-9
- TIMP-1

Lane A- Conditioned medium from *Non-loaded* FPCL

Lane B- Conditioned medium from *Endogenous static loaded* FPCL

Lane C- Conditioned medium from *Cyclical over loaded* FPCL
4.5.2. - REGULATION OF PROTEASES FROM SCAR FIBROBLASTS

Protease regulation from scar fibroblasts was monitored following non-loaded, endogenous static load and Low and High Aspect Ratio Cyclical Over Loaded FPCLs. Two scar specimens were used from the same patient (female, 39 years old), but from different sites (1) Abdomen and (2) Thoracotomy site.

4.5.2.1.- Endogenous contraction of Abdomen and Thoracotomy scar fibroblasts.

Endogenous contraction of abdomen and thoracotomy scar fibroblasts (39 years old, passage 4) was compared with normal fibroblasts (45 years old, passage 4) (FIG 4.5.6). The endogenous contraction forces produced by both abdomen and thoracotomy scar fibroblasts were similar. Peak contraction was between 6-10 hours, followed by a drop and plateau, and finally a second rise in contraction forces were monitored from 21 hours until the end of the experiment at 24 hours. Normal fibroblasts there was a steady increase in endogenous contraction forces throughout the 24 hours.

Endogenous contraction at 8 hours for abdomen scar fibroblasts was $98.13 \pm 6.4$ dynes, for thoracotomy scar fibroblasts it was $78.51 \pm 4.9$, and for normal fibroblasts it was $51 \pm 4.5$ dynes, giving a percentage difference at 8 hour contraction of 48% and 35% with abdomen and thoracotomy scar fibroblasts, respectively.
FIGURE 4.5.6.- Endogenous contraction forces generated from normal and scar fibroblasts, abdomen and thoracotomy, over 24 hours.

Dotted line represents contraction at 8 hours.

The rate of contraction analysis (FIG 4.5.7.) indicated that the greatest initial rate of contraction was produced by normal fibroblasts (48 dynes/hour) during the first 30 mins. The rate of contraction of both types of scar fibroblast rose steadily to their maximum at 2 hours (22.7 dynes/hour abdomen for fibroblasts and 27.4 dynes/hour for thoracotomy fibroblasts). This was followed by a slow and gradual reduction in contraction for scar fibroblasts until 5 hours. By 10 hours, the rates of contraction for both normal and abdomen scar fibroblasts were very similar.
FIGURE 4.5.7. - The rate of contraction generated from normal and mature scar fibroblasts, abdomen and thoracotomy, over first 10 hours of endogenous contraction.

Conditioned medium collected following non-loaded, Low Aspect Ratio (LAR) endogenous static load, LAR Cyclical Over Load (COL) and High Aspect Ratio (HAR) COL FPCLs from abdomen and thoracotomy scar fibroblasts were used for zymography (MMP-2 and MMP-9 only) and Western blot analysis. All protease responses were means of triplicate FPCL experiments. Abdomen and thoracotomy scar fibroblasts were used at passage 4.
4.5.2.2. Zymographic analysis

Total MMP-2 release rose significantly with abdomen scar fibroblasts, but was significantly decreased with thoracotomy scar fibroblasts following loading compared to the non-loaded lattice (FIG 4.5.8.).

With abdomen scar fibroblasts, total MMP-2 was increased by 6.5 fold (LAR static load) and 4.5 fold (LAR COL). However, using HAR COL MMP-2 was shown to have the same rate of release as the non-loaded lattice. In contrast, thoracotomy scar fibroblasts were observed to be substantially reduced following all 3 loading regimes. A decrease to 58% (LAR static load), 55% (LAR COL) and 28% (HAR COL) of MMP-2 release were measured in comparison to the non-loaded lattice.

Overall enzyme release rates from both types of scar fibroblasts were substantially higher in thoracotomy fibroblasts than abdomen fibroblasts. The rate of MMP-2 release was 20 fold greater from thoracotomy fibroblasts than abdomen fibroblasts in the non-loaded lattice. For both types of fibroblasts the predominant form of the enzyme was latent MMP-2 in all cases. Increases of 6.8 fold (LAR static load), 4.6 fold (LAR COL), and 1.3 fold (HAR COL) were measured in comparison to the non-loaded lattice in abdomen fibroblasts. However, a reduction of 67% (LAR static load), 58% (LAR COL), and 31% (HAR COL) were measured in comparison to the non-loaded lattice in thoracotomy fibroblasts.
FIGURE 4.5.8.-Comparison of MMP-2 release in medium conditioned for 24 hours during Non-loaded (FF gel), Endogenous static load (Endogenous contraction), Low Aspect Ratio Cyclical Over Load (LAR COL) and High Aspect Ratio Cyclical Over Load (HAR COL) of FPCLs with abdomen (A and B) and thoracotomy (C and D) scar fibroblasts.

Abdomen scar fibroblasts ** p ≤ 0.01 Low Aspect Ratio Cyclical Over Load vs. Non-loaded FPCL, *** p ≤ 0.001 Endogenous static load vs. Non-loaded FPCL.

Thoracotomy scar fibroblasts ** p ≤ 0.01 Endogenous static load and Low Aspect Ratio Cyclical Over Load vs. Non-loaded FPCL, *** p ≤ 0.001 High Aspect Ratio Cyclical Over Load vs. Non-loaded FPCL. (n=3)
As with MMP-2, total MMP-9 release was significantly increased with abdomen scar fibroblasts, but was significantly decreased with thoracotomy scar fibroblasts following loading regimes in comparison to the non-loaded lattice (FIG 4.5.9.).

For abdomen fibroblasts, total MMP-9 release rates were substantially increased with all loading regimes, with rises of 10 fold (LAR static load), 6 fold (LAR COL) and 12.6 fold (HAR COL) were measured in comparison to non-loaded lattices. However, enzyme release with thoracotomy fibroblasts was lowered following all loading regimes, to 50% (LAR static load), 80% (LAR COL), and 60% (HAR COL) of the non-loaded lattice enzyme release.

Like with MMP-2, the overall release of MMP-9 was greater with thoracotomy than abdomen fibroblasts. The rate of enzyme release for the non-loaded lattice was 22 fold greater with thoracotomy fibroblasts than abdomen fibroblasts.

For both scar fibroblast types active MMP-9 was the dominant form. For abdomen fibroblasts virtually all of the enzyme (> 90%) was present in the active form. Increases of 11 fold (LAR static load), 6 fold (LAR COL), and 14 fold (HAR COL) were measured in comparison to the non-loaded lattice. In contrast, active MMP-9 was greatly reduced with thoracotomy fibroblasts. It was decreased to 52% (LAR static load), 80% (LAR COL), and 61% (HAR COL) of that observed with the non-loaded lattice.
FIGURE 4.5.9.-Comparison of MMP-9 release rate in medium conditioned for 24 hours during Non-loaded (FF gel), Endogenous static load (Endogenous contraction), Low Aspect Ratio Cyclical Over Load (COL) and High Aspect Ratio Cyclical Over Load (HAR COL) of FPCLs using abdomen (A and B) and thoracotomy (C and D) scar fibroblasts.

Abdomen scar fibroblasts * p ≤ 0.05 Low Aspect Ratio Cyclical Over Load vs. Non-loaded FPCL, ** p ≤ 0.01 Endogenous static load and High Aspect Ratio Cyclical Over Load vs. Non-loaded FPCL.

Thoracotomy scar fibroblasts * p ≤ 0.05 Low Aspect Ratio Cyclical Over Load vs. Non-loaded FPCL, ** p ≤ 0.01 Endogenous static load and High Aspect Ratio Cyclical Over Load vs. Non-loaded FPCL. (n=3)
4.5.2.3.-Western blot analysis

A single sample of conditioned medium from each of non-loaded, LAR static loaded, LAR COL and HAR COL FPCLs were analysed by Western blotting. Fig 4.5.10. illustrates proteases detected by this method were mostly in their latent forms, but, some active forms were also observed.

MMP-1 was observed in its latent (55kDa) and active (45kDa) forms with both scar fibroblasts. For abdomen fibroblasts, MMP-1 increased following all loading regimes compared to the non-loaded lattice. Both forms of MMP-1 were measured following LAR static load and LAR COL, however, only latent MMP-1 was monitored following HAR COL. For thoracotomy fibroblasts MMP-1 was observed following non-loaded lattice contraction. Following all loading regimes MMP-1 was shown to decrease, with the largest reduction in MMP-1 release detected following HAR COL.

MMP-2 release was observed only in its latent form at 92kDa for both scar fibroblasts. For abdomen fibroblasts, there appeared to be a slight increase in MMP-2 following loading compared to non-loaded lattices. For thoracotomy fibroblasts, the only apparent rise in MMP-2 was demonstrated following LAR COL. In contrast, MMP-2 was apparently considerably reduced following LAR static load and HAR COL.

MMP-9 occurred in both latent (92kDa) and active (84kDa) forms for both scar fibroblasts, and latent and active MMP-9 forms were highly responsive to loading.
MMP-3 was detected in latent (55kDa) and active (45kDa) forms for both scar fibroblasts. With abdomen scar fibroblasts substantial rises in MMP-3 were observed following all loading regimes in comparison with the non-loaded lattice, this was greatest following LAR static load (with both latent and active forms present). However, only the latent form was seen following LAR COL and HAR COL. For thoracotomy fibroblasts, the largest bands for MMP-3 were seen following the non-loaded lattice and LAR static load (with both active and latent forms present). However, following LAR COL and HAR COL only latent MMP-3 was observed. Slight reductions in enzyme were seen following LAR static load and LAR COL, but only faint band was observed following HAR COL.

TIMP-1 activity was shown to be present at 28 kDa for both scar fibroblasts. With abdomen fibroblasts TIMP-1 decreased with loading and no activity was detected following HAR cyclical over loading. For thoracotomy fibroblasts moderate increases in activity were detected following all loading regimes compared to the non-loaded lattice.
FIGURE 4.5.10.-Western blot analysis of Non-loaded, LAR Static loaded, LAR Cyclical Over Loaded and HAR Cyclical Over Loaded conditioned media samples using abdomen and thoracotomy scar fibroblasts.

Key- CL - MMP-1
     GA - MMP-2
     ST - MMP-3
     GB - MMP-9
     TIMP-1

ABDOMEN SCAR FIBROBLASTS
Lane A- Conditioned medium from Non-loaded FPCL

Lane B- Conditioned medium from Low Aspect Ratio static loaded FPCL

Lane C- Conditioned medium from Low Aspect Ratio cyclical over loaded FPCL

Lane D- Conditioned medium from High Aspect Ratio cyclical over loaded FPCL

THORACOTOMY SCAR FIBROBLASTS
Lane E- Conditioned medium from Non-loaded FPCL

Lane F- Conditioned medium from Low Aspect Ratio static loaded FPCL

Lane G- Conditioned medium from Low Aspect Ratio cyclical over loaded FPCL

Lane H- Conditioned medium from High Aspect Ratio cyclical over loaded FPCL
4.5.3. - SUMMARY OF RESPONSES OF PATHOLOGICAL TISSUE FIBROBLASTS TO STATIC AND CYCLICAL LOADING REGIMES.

Protease and inhibitor release rates from pathological tissue fibroblasts, keloid and scars, were shown to be sensitive to all loading regimes in ways which differed in some respects from normal fibroblasts. Overall results from both keloid and scar fibroblasts are summarized in table 4.5.2. and 4.5.3.

Table 4.5.2.-Summary of keloid protease and inhibitor response to endogenous static load and cyclical over load. The arrows indicate the rise or fall of the protease or inhibitor in relation to the non-loaded lattice (↔).

**KELOID FIBROBLASTS**

<table>
<thead>
<tr>
<th>PROTEASE/INHIBITOR</th>
<th>NON-LOADED LATTICE</th>
<th>ENDOGENOUS STATIC LOAD</th>
<th>CYCLICAL OVER LOAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>↔</td>
<td>↑↑↑</td>
<td>↓</td>
</tr>
<tr>
<td>MMP-2</td>
<td>↔</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>MMP-3</td>
<td>↔</td>
<td>↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>MMP-9</td>
<td>↔</td>
<td>↑↑</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>↔</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>

**KEY**

↑/↓ slight increase/decrease

↑↑/↓↓ moderate increase/decrease

↑↑↑↑ substantial increase
Table 4.5.3.- Summary of scar protease and inhibitor response to endogenous static load and LAR and HAR cyclical over load. The arrows indicate the rise or fall of the protease or inhibitor in relation to non-loaded lattice (↔).

**ABDOMEN SCAR FIBROBLASTS**

<table>
<thead>
<tr>
<th>PROTEASE/INHIBITOR</th>
<th>NON-LOADED LATTICE</th>
<th>ENDOGENOUS STATIC LOAD</th>
<th>LAR CYCLICAL OVER LOAD</th>
<th>HAR CYCLICAL OVER LOAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>↔</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>MMP-2</td>
<td>↔</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>MMP-3</td>
<td>↔</td>
<td>↑↑↑</td>
<td>↑↑</td>
<td>↑↑</td>
</tr>
<tr>
<td>MMP-9</td>
<td>↔</td>
<td>↑↑</td>
<td>↑↑</td>
<td>↑↑↑</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>↔</td>
<td>↓</td>
<td>↓↓</td>
<td>*</td>
</tr>
</tbody>
</table>

**THORACOTOMY SCAR FIBROBLASTS**

<table>
<thead>
<tr>
<th>PROTEASE/INHIBITOR</th>
<th>NON-LOADED LATTICE</th>
<th>ENDOGENOUS STATIC LOAD</th>
<th>LAR CYCLICAL OVER LOAD</th>
<th>HAR CYCLICAL OVER LOAD</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-1</td>
<td>↔</td>
<td>↓</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
<tr>
<td>MMP-2</td>
<td>↔</td>
<td>↓↓</td>
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<td>↓↓</td>
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<tr>
<td>MMP-3</td>
<td>↔</td>
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<tr>
<td>MMP-9</td>
<td>↔</td>
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<td>↓↓</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>↔</td>
<td>↓↓</td>
<td>↓↓</td>
<td>↓↓</td>
</tr>
</tbody>
</table>

**KEY**

↑/↓ slight increase/decrease

↑↑/↓↓ moderate increase/decrease

↑↑↑/↓↓↓↓ substantial increase

"*" denotes detection below the limits of the technique used
(A) Endogenous contraction forces of keloid fibroblasts (from a range of sites) were demonstrated to be considerably higher than normal fibroblasts. Scar fibroblasts from different locations within the same individual demonstrated marked changes in their contraction forces.

(B) The overall rate of enzyme release, for keloid fibroblasts, of MMP-1 and MMP-9 were shown to increase following static and cyclic loading, whereas, with MMP-2 and MMP-3 rates of release were decreased. No change in TIMP-1. These protease changes were similar to those of normal fibroblasts.

(C) For both types of scar fibroblast protease responses differed with each site following loading. Protease responses from abdomen fibroblasts were generally increased, and reduced responses were observed with thoracotomy fibroblasts.
CHAPTER 5 - DISCUSSION

Wound healing in the skin and internal organs involves a series of overlapping events requiring extensive communication amongst similar and different cell types through several chemical mediators (Clark, 1996). Of the many cell types, the presence of the fibroblast is crucial and central to healing. A dynamic relationship occurs between fibroblasts and the surrounding ECM. The integrity of the ECM is controlled by the equilibrium between synthesis and local degradation of its different components (Ries and Petrides, 1995). Fibroblasts make and maintain the ECM through synthesis of its components, deposition of collagen, and remodelling of the matrix using proteases (Kurkinen et al., 1980; Welch et al., 1990). Interactions between fibroblasts and the matrix, mediated by integrins, determine some of the cell’s properties. These include morphology, apoptosis, proliferation, expression of growth factors and receptors, migration and synthetic ability including extracellular proteases such as MMPs (Mauch et al., 1988; Grinnell, 1994; Clark et al., 1995).

One property of dermal fibroblasts, is their ability to contract a 3-D gel of type I collagen. By adhering and bundling together collagen fibres a dense tissue-like structure can be formed rather than a network of loose fibres (Bell et al., 1979). During these interactions with collagen fibres, fibroblasts have been shown to change their morphology from stellate to bipolar cells (Bell et al., 1979; Bellows et al., 1981; Stopack and Harris, 1982; Bellows et al., 1982).
Recent studies by Brown et al. (1998) and Eastwood et al. (1998) have found that dermal fibroblasts within a 3-D matrix generate a stable, natural tension. Maintenance of this tension is regulated by fibroblasts through their interactions with the matrix.

The aim of this study was to test whether and how protease production is linked to external matrix loading. Alterations of proteases in response to mechanical loading would be predicted as some cells have been shown to penetrate the collagen matrix during its contraction, a process for which degradation of the matrix maybe necessary (Grinnell, 1994; Occleston et al., 1994).

Evidence for possible protease activity in FPCLs has come from morphological studies which have identified cavities within the lattice surrounding cells. However, these could have been produced by disruption of inter-fibrillar interactions as a result of cell locomotion through the lattice rather than collagen lysis (Mazure and Grierson, 1992; Porter et al., 1998).

The mechanisms of regulation of MMP activity during FPCL contraction are controversial. However, cells in contact with ECM components on 2-D or within 3-D substrates have been found to produce more MMPs in comparison to cell monolayers (Woodley et al., 1987; Mauch et al., 1989; Azzam and Thompson, 1992; Lambert et al., 1992; Hunt et al., 1993; Saarialho-Kere et al., 1993; Seltzer et al., 1994; Tremble et al., 1994; Partridge et al., 1997; Tomasek et al., 1997; Hass et al., 1998).
During this investigation FPCLs were subjected to nil-tension (non-load), endogenous static loads and exogenous loading regimes. The results have demonstrated that MMP-1, MMP-2, MMP-3, MMP-9, uPA, tPA and TIMP-1 were released into the media during FPCL contraction and loading. Furthermore, the rate of release was shown to be altered differentially for each protease and inhibitor.

5.1. - ENDOGENOUS STATIC LOAD AND CYCLICAL LOADING.

During this study FPCLs were subjected to non-load, endogenous static load, and exogenous cyclical loading regimes. The forces applied to the lattices during loading regimes were designed to be physiological in magnitude, i.e. in relation to the cells and their low density collagen matrix. Hence, they were comparable to forces generated by the cells and to the forces that the FPCLs respond to (Brown et al., 1998; Eastwood et al., 1998). The cyclical loading was of low frequency (for 1 hour at $2.28 \times 10^{-4}$ Hz), and strain (approximately 0.02% on the cells) but, within this weak, random collagenous substrate, cells were clearly sensitive to such modest loading. This was demonstrated by alterations in their protease and inhibitor responses after application of endogenous and exogenous loading regimes to FPCLs compared to non-loaded lattices.

The changes in protease activity found here were similar to those of previous studies. These investigations have indicated that fibroblasts could be induced by chemical and physical methods to produce proteases by cell cytoskeletal changes or by changes in the mechanical environment of cells. In this study, MMP-1 was considerably increased
following static and exogenous loading regimes compared to non-loaded FPCLs. Other groups have found an increase in MMP-1 in FPCLs compared to monolayers (Mauch et al., 1989; Lambert et al., 1992). Higher levels of MMP-1 were measured in non-loaded lattices than in static loaded (tethered lattices) (Unemori and Werb, 1986; Lambert et al., 1992). These differences in MMP-1 levels can be attributed to the type of non-loaded lattice models used by these investigators, and variations in MMP-1 measurements.

During the Unemori and Werb investigation, mechanical stress, using a stress-relaxation FPCL model, was allowed to develop within the FPCLs (with $2 \times 10^5$ rabbit synovial fibroblasts) for 24 hours before the lattices were released. MMP-1 measurements followed the release of these stress-relaxed lattices at 24 hours and were compared to tethered (static loaded) lattices. MMP-1 activity was shown to be greater in stress-relaxed lattices, released at 24 hours, and this proceeded to increase up to 48 hours. However, the Lambert et al. investigation has shown that there was no difference in the amount of MMP-1 between non-loaded and tethered lattices until day 5 (with $6 \times 10^5$ dermal fibroblasts in each lattice). At day 5, MMP-1 was shown to be two fold higher in non-loaded lattices than tethered lattices. The accumulated MMP-1 activity in these lattices was shown to increased with time. The above investigations cannot be directly compared to the current study as (I) all non-loaded lattices in this investigation were untethered/released from the culture well at the start of the experiment, without allowing stress to build-up within the lattice. (II) results here have
been calculated as a rate and not as an accumulation with time. The greatest rate of
protease release occurred at 8 hours in the current investigation, and this was shown to
decrease over 24 and 48 hours. Subsequently, all protease release rates compared
conditioned medium on completion of the loading regimes at 24 hours.

Factors that cause a change in cell shape are thought to be one of the major
determinants for MMP expression for fibroblasts. For instance, changing rabbit
synovial fibroblast morphology with cytoskeletal depolymerising agents, such as
cytochalasin-B, cytochalasin-D, and 12-tetradecanoyl-phorbol-13-acetate (TPA) (a
phorbol diester tumour promoting agent) induced MMP-1 production (Aggelar et al.,
1984). Similarly, using cytochalasin-B or TPA, Werb et al. (1986) found co-ordinate
synthesis of latent MMP-1 and MMP-3 correlating with an alteration in the actin
cytoskeleton, also in rabbit synovial fibroblasts. In this study, dermal fibroblasts were
shown to change their morphology with loading regimes. In non-loaded lattices,
fibroblasts had a stellate appearance with short processes, whereas after static and
cyclical loading they became bipolar, similar to changes observed by Bell et al. (1979),
Bellows et al. (1981), Stopack and Harris (1982), and Bellows et al. (1982). Cells
within static and cyclical loaded lattices had the longest processes, and it was within
these lattices that most MMP-1 was observed by immunostaining, suggesting that
changes in cell shape were linked to MMP synthesis. Therefore, MMP-1 is synthesised
by fibroblasts in lattices in increasing amounts following loading, probably due to
changes in cell shape.
Following non-load, static load, and cyclical loading regimes of FPCLs protease production was shown to be altered. These changes may be mediated by cell-matrix interactions involving matrix degradation, changes at cell attachment-detachment sites, and during cell movement.

5.1.1. - Matrix Degradation

MMP-1 Grinnell, (1994) and Occleston et al. (1994) have suggested that contraction of FPCLs is linked with MMP activity. The principle protease involved during collagen degradation is MMP-1. It is the only enzyme capable of degrading triple helical collagen chains. During FPCL contraction MMP-1 levels would be expected to increase if lysis of the lattice is required for contraction. MMP-1 has been shown to increase during lattice contraction, whilst type I collagen synthesis was down regulated (Nusgens et al., 1984; Unemori and Werb, 1986; Mauch et al., 1988 and 1989; Seltzer et al., 1994). MMP-1 was regulated at the transcriptional level, whereas, collagen synthesis was regulated by transcriptional and by post-transcriptional mechanisms (Eckes et al., 1993; Langholz et al., 1997). The rate of contraction during the first 8 hours in this investigation suggests MMP-1 may have an important role to enable cell invasion and motility within the collagen matrix, but this has not been investigated and would need to be confirmed. MMP-1 was shown to be present mostly in the latent form during non-loaded and static loaded lattices. Its levels were increased during cyclical loading, and both latent and active forms were observed, suggesting that matrix lysis may have
increased with cyclical loading. Following initial matrix lysis, by MMP-1, it is possible for other proteases to continue matrix degradation.

**MMP-3** In this study, intracellular MMP-3 was found to be present in FPCLs following all loading regimes. However, MMP-3 release measured in the conditioned media was found to decline following cyclical loading. This suggests that extracellular MMP-3 was not required by cyclically loaded cells. Alternatively, MMP-3 could be regulated in part by mechanical stimulation to act as an activator for other latent MMPs, particularly MMP-1 and MMP-9. MMP-3 was observed to decrease with cyclical loading, while MMP-1 and MMP-9 increased with loading. The inverse correlation could be due to the use of MMP-3 to activate the other enzymes, leading to overall matrix lysis.

**MMP-2 and MMP-9** Both enzymes are known to efficiently degrade denatured collagens (Mignatti et al., 1996), and as such it is conceivable that they may have been used for further matrix degradation initiated by MMP-1.

**PLASMINOGEN ACTIVATORS** The plasminogen activators, uPA and tPA, activate plasminogen to plasmin, which in turn activates latent MMPs primarily latent-MMP-1 and latent-MMP-3. Plasmin can degrade denatured collagen and other matrix components. Hence, plasminogen activators could contribute to matrix degradation.
TIMP-1 In this study TIMP-1 was found to decrease with cyclical loading, in contrast, MMP-1 production rose during cyclical loading. This suggests that cyclical loading could tend to push the MMP-1 and TIMP-1 ratio to favour lysis of the collagen matrix.

5.1.2. - Cell attachment-detachment

A recent study on the mechanical responses of fibroblasts in FPCLs to exogenous loading has found that cells constantly adjusted the matrix tension in response to the loading (Brown et al., 1998). Increased external loading was immediately followed by a reduction in cell-mediated contraction, whilst, decreased external loading elicited increased contraction. Apparently these changes in cell-mediated contraction occurred through attachment/detachment of collagen fibres.

Attachment/detachment of collagen fibres is mediated via formation of adhesion plaques and integrins (α1β1 and α2β1)(Yamada et al., 1996; Horwitz, 1997). α2β1 has been shown to be selectively upregulated in fibroblasts during contraction of collagen lattices (Klein et al., 1991). During endogenous and exogenous loading regimes cell adhesions need to be constantly attached/detached and this may involve MMPs. Werb et al. (1989) have demonstrated that the interaction of synovial fibroblasts with fibronectin via α5β1 (fibronectin receptor) was followed by a strong induction of MMP-1 and MMP-3 synthesis. Partridge et al. (1997) have shown proteolytic activation of MMP-2 and MMP-9 by endothelial cells occurred mainly at focal contacts. Focal contact extracts were shown to contain MMP-3, which may have
further influenced MMP-9 activation at this site. Recently, Hass et al. (1998) have suggested mechanical stimuli and specific ligand-matrix interactions are necessary for achieving MMP induction. These investigations indicate that cells are capable of concentrating and activating MMPs at specific sites on their surfaces for focused degradation of ECM components.

5.1.2.1. - Role of MMP-2 and MMP-9 and a “Traction-Maintenance Hypothesis”

During this study, dramatic increases in MMP-9 were measured following cyclical loading, 18 fold and 37 fold depending on the type of cyclical loading, and majority of this enzyme was present in the active form. At the same time there was little change in MMP-2. As there did not seem to be excessive lysis of the lattice, the question arises as to the roles of MMP-2 and MMP-9 during endogenous and exogenous loading regimes? Generation of tractional forces appear to be related to cell attachment and motility, and the importance of cell attachment and motility to FPCL contraction has been demonstrated in the CFM model (Eastwood et al., 1996; Sethi et al., 1997; Brown et al., 1998; Eastwood et al., 1998). This attachment-dependence of fibroblasts during contraction suggests a potential functional application for MMP-2 and MMP-9 during FPCL contraction and in some aspects of cell mobility. Grinnell (1994) and Porter et al. (1998) have found evidence of collagen reorganisation, bundling, and channel formation during FPCL contraction, which suggests involvement of MMPs as matrix degradation is likely to have taken place for these processes to have occurred.
Cells which contract non-loaded lattices appear to constantly draw relatively fresh, unused collagen towards themselves by bundling the collagen (Grinnell, 1994), producing the characteristic shrinkage-contraction. These collagen fibrils will be largely "fresh" and untouched by other cells. This feature of matrix shrinkage is not found in static, loaded lattices which do not significantly shrink, hence, there can be very little drawing-in of fresh collagen. This will be more pronounced for cells in cyclically loaded lattices where cells will be obliged to move constantly backwards and forwards over the same cyclically moving area of matrix. Cells will then be forced to make and break attachments to the same collagen fibres during each cycle. The process of cell movement involves constant formation of new attachment plaques, involving integrin and fibronectin/vitronectin complexes bound to collagen, and proteolytic disruption of these attachment plaques (Chapman, 1997), to allow constant attachment/detachment of collagen fibres by fibroblasts following cyclical loading.

Contamination of the substrate by accumulation of fragmented binding proteins (i.e. fibronectin and vitronectin) and integrin fragments (i.e. non-functional debris from attachment plaques) would gradually restrict normal cell attachment. This would then lead to loss of traction and a failure to produce mechanical-contractile responses in the FPCL. The presence of "attachment debris" would alter the surface attachment properties of the collagen substrate. This is consistent with recent work on fibroblast locomotion (Palecek et al., 1997) indicating that there are optimal levels of cell-substrate adhesion. For instance, if adhesion is too strong or too weak cell motility is
reduced. It would therefore seem to be a necessary part of normal contractile and motile activity that attachment plaque contamination is removed from a substrate, to “unblock” attachment/receptor sites of matrix debris prior to cell re-attachment. This would be particularly true when cells repetitively uses the same region of matrix, as in cyclical loading.

Suitable candidates for a “traction-maintenance” role (or contamination cleansing) would be MMP-2 and MMP-9 given their range of non-collagenous substrates (Murphy and Reynolds, 1993; Nagase, 1997). These enzymes would leave intact collagen fibrils unchanged, but, remove non-collagenous proteins (fibronectin, vitronectin, integrin fragments) or denatured collagen. It is hypothesized that the functions of MMP-2 and MMP-9 are to clean away accumulations of attachment proteins and lyse attachment plaques from the collagen substrate during exogenous loading. The result of this would be to regenerate the attachment surface (in this case collagen) by a process analogous to polishing. It is interesting in this context that the increase in MMP-9 in this system was almost all active enzyme. This suggests that an alternative pathway for “traction maintenance” had been activated, producing activatable enzyme.
5.1.3. - Cell mobility and Plasminogen activators.

Cellular movement over/through an ECM substratum involves a number of factors, these include (I) a stimulus for movement, (II) changes in integrins and adhesion plaques, (III) matrix degradation to allow cell movement, and (IV) changes in the cell cytoskeleton. MMPs have a role in some of these processes for example, MMP-2, MMP-3 and MMP-9 have been located at focal contacts (Partridge et al., 1997). Initial matrix degradation may involve MMP-1, followed by participation by other MMPs and plasminogen activators (Murphy and Reynolds, 1993; Mignatti et al., 1996; Nagase, 1997).

The plasminogen activators, uPA and tPA, have been shown to have different physiological roles. uPA is strongly implicated in (a) activation of latent forms of MMPs and (b) in cellular motility, and tPA is involved with clot lysis (Mignatti et al., 1996; Chapman, 1997). uPA has been shown to have two effects on cell motility-proteolytic and non-proteolytic (Andreasen et al., 1997). The proteolytic mechanism of uPA enhancement on cell migration would involve plasmin generation at focal adhesion sites at the cell edge, leading to ECM degradation and enabling detachment of the trailing edge. In the non-proteolytic mechanism, uPA would stimulate cell migration by enhancing adhesion at the leading edge, through stimulation of binding of uPA-receptor to vitronectin, modulation of uPA-receptor/integrin interactions, and/or by initiation of signal transduction cascades. Evidence for these mechanisms has come from (a) up regulated expression of uPA detected at the leading cell edge during
migration of several cell types, (b) inhibition of migration by antibodies against uPA, (c) addition of plasminogen and plasmin activity enhancing cell migration, and (d) enzymatic inactive uPA variants stimulating migration of different cell types (Andreasen et al., 1997).

In this study, uPA was increased with static loading and not detected with cyclical loading, whilst tPA increased with both loading regimes. It seems possible that uPA may have been involved with increased cell motility during static loading, and during cyclical loading its role may have been altered. It may have been used intracellularly by fibroblasts to modulate uPA-receptor/integrin interactions. These interactions could further lead to activation of MMP-2, MMP-3 and MMP-9 found at focal contacts (Partridge et al., 1997). From this study, it seems possible that during cyclical loading cells switched from secretion of uPA to tPA. It is tempting to suggest that the fall in uPA and increase in tPA following cyclical loading represents cells switching to a previously redundant, but, parallel pathway replacing the function of uPA during cell migration. Hence, in this study, tPA may have a more central role in cell invasion/motility of the lattice and degradation of the collagen matrix, i.e. by enabling cells to channel through the matrix as reported previously (Porter et al., 1998).

The changes within fibroblasts, at the leading edge and at the focal contacts, remain uncertain at present and require further detailed investigations of cell motility studies. Such as by video time-lapse microscopy, inhibition studies and immunolocalisation.
5.2. - EFFECTS OF INCREASING FIBROBLAST SOURCE-TISSUE AGE WITH STATIC AND CYCLICAL LOADING.

In this study, dermal fibroblasts from increasing source-tissue age groups (mean age of groups 15, 30, 45 years old, and 60 year old specimen) were found to have considerably different contractile properties when comparing initial/maximum rate of contraction, peak force, and maximum force generated. These properties changed dramatically in relation to increased age of an individual.

With increasing age groups (15-45 years old) fibroblast endogenous contraction forces were demonstrated to decrease. However, a rise in contraction force was monitored with the 60 year old specimen. The results from this study are in keeping with previous investigations where fibroblasts from young source-tissue age were found to be highly contractile in untethered 3-D collagen lattices. Marks et al. (1990) have demonstrated that lattices constructed with sun exposed fibroblasts contracted more than lattices with sun protected fibroblasts. The difference in lattice contraction from these sites was greatest in younger patients, with contraction decreasing in a linear manner as the age of the patients increased (49 to 74 year old). Kono et al. (1990b) have demonstrated the influence of ageing on wound healing by evaluating the relationship between ageing and dermal fibroblasts to contract untethered lattices. Fibroblasts from children (0-15 year old), an adolescent group (16-40 year old), a middle aged group (41-60 year old) and an elderly group (61 year old and older) were used in the study. Gel contraction at 10th passage was demonstrated to be greatest with
the childrens group, whilst contraction was shown not to differ among the other groups. By 30th passage, there was no difference in fibroblast contraction between all groups. Indicating that younger fibroblasts were more contractile than the older fibroblasts, and were readily influenced by in vitro ageing.

Protease profiles (only MMP-2 and MMP-9 monitored) from non-loaded, static load and cyclical loading were found to be reproducible within each of the age groups. Interestingly, the highest enzyme releases (MMP-9) were associated with the most contractile groups (15 and 60 year old), i.e. generating the greatest maximum force. This suggests that FPCL contraction was linked with fibroblast contractile properties and is dependent on protease production. From this study, it may be possible to use the protease profile demonstrated here as a measure of fibroblast mechano-activation.

The difference in contractile ability between the groups suggests that wound healing could be retarded, i.e. wounds in older individuals heal slower. This has been found from reports as early as 1916, which have claimed that wound healing was impaired with human ageing (DuNouy, 1916). DuNouy followed the rate of open wound closure in soldiers of different ages (20-40 years old) during World War I and concluded that the younger individuals required a shorter time for wound contraction and closure. Hence, the results from this study are important not only for normal healing as people become older, but also in age associated diseases, such as premature
ageing syndromes- Werner syndrome, progeria (Hutchinson-Gilford) syndrome, or Cockayne syndrome (Millis et al., 1992).

5.3. - EXOGENOUS CONTINUOUS LINEAR LOADING.

In these experiments, protease responses were measured following exogenous continuous linear loading regimes, with three forms of continuous linear loading (10 mins, 1 hour, and 11 hours) and 1 form of continuous linear unloading (11 hours). The overall responses for all protease and inhibitor release following all types continuous loading/unloading regimes were similar, but different from endogenous static loading. The greatest changes in enzyme production (up or down) were initiated by the total length of the change (i.e. time the cells were under a changing load/unload), but not the change in the rate of strain (i.e. total force) applied to FPCL. Additionally, protease responses here are consistent with previous sections, with substantial increases in tPA and MMP-9, suggesting that fibroblasts use tPA and MMP-9 for processes of matrix invasion and attachment/detachment of receptor sites during responses to the application of load. Therefore, fibroblasts were able to perceive continuous linear load/unloads (with different forces and duration times) and distinguish between them.

Previous investigations have illustrated that loading regimes (static, continuous and cyclical loads) elicit many cellular responses, including significant increases in cardiac endothelial cell proliferation (Widmann et al., 1992), the expression of the genes for the extracellular matrix proteins, types I, III, and IV collagens, fibronectin and laminin
(Yasuda et al., 1996), MMP-1 production (Carano and Siciliani, 1996), activation of the c-Jun NH$_2$-terminal kinase and extracellular signal-regulated kinase cascades (MacKenna et al., 1998). However, it is important to note that all of these studies used large strain and high frequency mechanical loading regimes relative to this investigation, and the forces applied were not physiological for cells in a weak or monolayer substrate.

5.4. - LOW ASPECT RATIO VS. HIGH ASPECT RATIO LATTICE CONFIGURATION.

The previous sections have shown how protease production was altered following endogenous static load and exogenous loading regimes (cyclical and continuous loads). All of these experiments were performed with the FPCL attached to the CFM or t-CFM instruments in a Low Aspect Ratio (LAR) lattice configuration (ratio of length to width). In this section, protease production was monitored following static and cyclical loading of LAR and HAR lattice configurations. The results have demonstrated that the overall protease and inhibitor profiles were similar for both lattice configurations (and previous sections). But in all cases exaggerated responses (up or down) in the protease-inhibitor profiles were found with HAR lattices and cyclical loading. Therefore, HAR lattice configuration further activated fibroblast mechano-responses, and this response was strain sensitive.
Recent studies by Eastwood et al. (1998) have mapped out the pattern of strain developed in FPCLs during contraction and loading. The greatest strains were shown to be produced within the HAR lattice configurations, and were a consequence of lattice orientation. The collagen lattice orientation was shown to dictate the mechanical forces and cellular activity of fibroblasts within these lattices.

Examination of whole HAR lattices in this study have shown that fibroblasts were aligned in the direction of maximum strain. The effect of these strains were shown to change the morphology of the cells within the lattice from stellate to long bipolar cells. The results from this investigation are in agreement with the findings of Eastwood et al. (1998) i.e. cellular alignment occurred along lines of strain in HAR lattices.

Immunolocalisation studies are required to detect whether changes in the cell cytoskeleton were parallel with intracellular MMP distribution. Recently, Tomasek et al. (1997), have suggested that the ECM and actin cytoskeleton transduce signals that modulate MMP-2 activation and regulate tissue remodelling. This was confirmed by Partridge et al. (1997), who have shown that actin depolymerisation triggered MMP-mediated degradation of collagen. These investigations indicate that alterations in the organisation of the actin cytoskeleton could promote production of MMPs by fibroblasts in lattices.
Cells within the body are subjected to various forms of mechanical loading, including the tangential shear stress due to fluid flow and normal stress due to pressure as well as to other mechanical stimuli such as tensile stress from neighbouring cells, ECM, and the internal tension of the cytoskeleton (Shyy and Chien, 1997). These mechanical stresses play important roles in physiological processes, such as cell growth, differentiation and apoptosis. Previous studies of stress on FPCLs were shown to affect collagen expression (Carver et al., 1991), induction of MMP-1 (Lambert et al., 1992), cyclical AMP pathway (He and Grinnell, 1994), and tenascin C production (Chiquet-Ehrismann et al., 1994). Recently, Maniotis et al., (1997) and MacKenna et al., (1998) have suggested that integrins act as mechano-receptors and transmit signals to the cytoskeleton, mediated simultaneously at multiple locations inside the cell through a tensionally integrated cytoskeleton. It is possible that distortion of the cytoskeletal framework would then result in altered cellular responses, as seen here following mechanical loading of FPCLs and lattice re-orientation.

Recently, Ingber (1998) demonstrated that the structure of the cell’s cytoskeleton could be changed by altering the balance of physical forces transmitted across the cell surface. This would be influential as enzymes, protein synthesis, energy conversion and growth processes are affected by the cytoskeleton. In this study, mechanical tensioning of FPCLs was shown to influence cellular responses. Here protease and inhibitor production was demonstrated to be greatly affected by these forces, some proteases were shown to be highly sensitive to loading and subsequently their
production increased (MMP-1, MMP-9 and tPA), whilst other proteases decreased their production (MMP-3 and uPA), or remained mostly unchanged (MMP-2).

5.5. - STATIC AND CYCLICAL LOADING OF PATHOLOGICAL TISSUE FIBROBLASTS.

To appreciate how mechanical stimulation effects normal wound healing, it is also necessary to understand how external loading influences pathological tissue repair.

5.5.1. - Contractile forces.

It has been suggested that activated fibroblasts in keloid and hypertrophic scars are unable to degrade collagen, and consequently their ability to remodel the pre-existing, randomly orientated collagen matrix into a more uniform, organised matrix is impaired (Tredget et al., 1997). *In vitro* studies comparing contraction of normal fibroblasts with those derived from hypertrophic and keloids in FPCLs have produced mixed results. Some have found increased contractility of pathological fibroblasts (Sahara et al., 1993; Garner et al., 1995), whilst, others have shown no difference (Ehrlich, 1988b; Nedelec et al., 1995; Sato et al., 1996). In this study, keloid fibroblasts were found to be “hyper-contractile”, as they generated exceptionally high contractile forces compared to normal fibroblasts. These high forces were reproduced from keloid fibroblasts cultured from a number of sites from different individuals. In contrast,
contraction forces produced from scar fibroblasts (abdomen and thoracotomy) were found to differ considerably from each site, and even within the same individual when compared with normal fibroblasts. This suggests that the contractile properties of these pathological tissue fibroblasts were substantially different to each other and to those of normal fibroblasts.

5.5.2. - Protease activity.

Previous studies have found that MMP-1 levels were reduced on stimulation of keloid and hypertrophic scar fibroblasts (Abergel et al., 1985; Ghahary et al., 1995). However, these findings are not consistent as Cohen et al. (1971) and McCoy and Cohen (1982) produced increased MMP-1 activity from keloid, hypertrophic scar and mature scar fibroblasts. The findings of this current study are mainly in agreement with the latter investigations. MMP-1 produced by keloid fibroblasts increased with static loading, but, was decreased following cyclical loading. However, with scar fibroblasts results were variable, abdominal scar fibroblasts synthesised more MMP-1 during static and cyclical loading, whereas, MMP-1 levels decreased for thoracotomy scar fibroblasts. This suggests that pathological tissue fibroblasts not only differ in their protease responses in relation to normal fibroblasts, but, also between locations on the same individuals.
5.6. - CLINICAL IMPLICATIONS.

The involvement of mechanical stresses in the wound is demonstrated by the stretching of dermal scars and extended scarring resulting from increased skin tension (Arem et al., 1976; Sommerlad and Creasey, 1978; Burgess et al., 1990; Meyer and McGrouther, 1991).

The results from this study have raised the question, “Can excessive matrix formation following injury and surgery be controlled using protease management programmes?” Equally can we use the protease profiles to design mechanical scar management regimes.

Protease management regimes have potential applications for reducing scar contraction and increasing scar remodelling. External mechanical stimulation could influence scar remodelling by placing the region under tension, i.e. by initial suturing of the wound and subsequent physiotherapy. Mechanical stimulation may reduce connective tissue accumulation by increasing secretion of matrix degrading proteases. Consequently, stretching of scars could lead to structural reorganisation, repair and remodelling of scar connective tissue following injury or surgery.
5.7. - FUTURE WORK

From this study a number of interesting questions have been raised for further work. These include-

(a) To demonstrate how fibroblast contractile properties are affected following protease inhibition.

(b) To establish the roles of uPA and tPA by detailed cell motility studies.

(c) To establish whether changes in proteases and the cell cytoskeleton are parallel with lines of strain within the lattice.

(d) To establish whether information from mechanical loading is passed via integrins.

By inhibiting integrins and observing the protease response.

(e) To monitor how collagen synthesis changes in response to mechanical loading, and if the responses are inversely related to protease production.
5.8. - CONCLUSION

In conclusion, the results from this investigation have shown that protease and inhibitor production in fibroblasts is linked to endogenous and exogenous mechanical stimulation of FPCLs. Also, fibroblasts have the ability to distinguish and respond to each stimulation differentially by altering their release rates. These changes can be related to the control of cell-matrix attachment, locomotion and contraction of the matrix.

The release of proteases and inhibitor following mechanical stimulation of FPCLs are not isolated events as they are affected by other factors. Mechanical loading of lattices may initiate a change in the nuclear response, through cell cytoskeleton distortion and signal transduction pathways to promote cell attachment/detachment via integrins and by modifying the secretion of cytokines, and proteases and inhibitors. Hence, these factors are all interdependent.
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