Cellular and molecular basis of wound healing: Effects of growth arrest induced by antimetabolites on ocular fibroblast behaviour

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This thesis is dedicated with love to my wonderful wife Louise. Thanks for your help and support throughout it all. With you anything is possible.

"The highest reward for a person's toil is not what they get for it, but what they become by it" - John Ruskin
Abstract

The wound healing response, in which the fibroblast is a key player, is a major cause of clinical morbidity throughout the human body. This response is of particular importance in the eye, as it plays a role in the pathogenesis or failure of treatment of many visually disabling or blinding conditions in the world today. An example of this is the failure of the surgical treatment of glaucoma due to excessive scarring. Previous studies have shown that single exposures to antimetabolites result in the long term growth arrest of ocular fibroblasts \textit{in vitro}, and reduced scarring \textit{in vivo} following glaucoma surgery. The overall effects of these treatments on fibroblast wound healing behaviour are not known. Additionally, growth arrested fibroblast feeder layers have been routinely used to serially cultivate keratinocytes for over twenty years, the mechanisms underlying this support of keratinocyte growth being unclear. This thesis investigated the effects of single exposures to antimetabolites on molecular and cellular aspects of ocular fibroblast wound healing behaviour.

Growth arrested ocular fibroblasts were found to produce a number of regulatory molecules both at the message and protein levels using a quantitative reverse transcriptase polymerase chain reaction technique and enzyme linked immunosorbent assays. The molecules produced included: growth factors (transforming growth factor beta-1 and basic fibroblast growth factor); growth factor receptors (transforming growth factor beta type II receptor, fibroblast growth factor receptor and epidermal growth factor receptor) ; and extracellular matrix molecules (collagen type I, collagen type III and fibronectin) up to 48 days post-growth arrest. Fibroblasts were also found to migrate and contract collagen following growth arrest. Additionally, matrix metalloproteinases were identified as a novel, essential and possibly ubiquitous component of fibroblast-mediated collagen contraction.

The experiments in this thesis have led to an increased understanding of the biology of growth arrest \textit{in vitro}, and may explain how growth arrested fibroblasts support keratinocyte growth \textit{in vitro}. Additionally, these findings may have clinical implications with respect to the modulation of scarring \textit{in vivo}. Finally, the identification of a novel and potentially ubiquitous mechanism of collagen contraction may lead to the development of new anti-scarring strategies.
All of the experiments in this thesis were carried out personally, unless otherwise stated.

The experiments were conceived and designed by myself, after discussions and guidance from my supervisors.
## Chapter 1: INTRODUCTION

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<td>ELISA quantitation of MMP and TIMP proteins produced during collagen contraction</td>
</tr>
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<td>3.4</td>
<td>LDH release into overlying medium during collagen contraction, following exposure to MMP inhibitors</td>
</tr>
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</tr>
<tr>
<td>200</td>
<td>3.6</td>
<td>Attachment of cells to collagen in the presence of MMP inhibitors</td>
</tr>
</tbody>
</table>

**List of Abbreviations**

- **ANOVA** - analysis of variance
- **APMA** - aminophenylmecuric acetate
- **BP** - binding protein
- **BSA** - bovine serum albumin
- **CFM** - culture force monitor
- **DNA** - deoxyribonucleic acid
- **cDNA** - complimentary DNA
- **DMEM** - Dulbecco's modified Eagles medium
- **DMSO** - dimethysulphoxide
- **ECM** - extracellular matrix
- **EGF** - epidermal growth factor
- **ELISA** - enzyme linked immunosorbent assay
- **EDTA** - ethylenediamine tetra-acetic acid
- **FCS** - foetal calf serum
- **FGF** - fibroblast growth factor
- **bFGF** - basic fibroblast growth factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>5FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>aFGF</td>
<td>acidic fibroblast growth factor</td>
</tr>
<tr>
<td>LTGFβ</td>
<td>latent transforming growth factor beta</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen activated protein kinase</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium-bromide</td>
</tr>
<tr>
<td>MMC</td>
<td>mitomycin-C</td>
</tr>
<tr>
<td>MT-MMP</td>
<td>membrane type matrix metalloproteinase</td>
</tr>
<tr>
<td>PUMP-1</td>
<td>putative metalloproteinase-1</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>QCRT-PCR</td>
<td>quantitative competitive reverse transcriptase polymerase chain reaction</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RRA</td>
<td>radioreceptor assay</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcription / transcriptase</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SE</td>
<td>standard error</td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscopy</td>
</tr>
<tr>
<td>TGFα</td>
<td>transforming growth factor alpha</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of matrix metalloproteinases</td>
</tr>
<tr>
<td>tPA</td>
<td>tissue type plasminogen activator</td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscopy</td>
</tr>
<tr>
<td>u-PA</td>
<td>urokinase plasminogen activator</td>
</tr>
<tr>
<td>vol</td>
<td>volume</td>
</tr>
<tr>
<td>WST</td>
<td>4-[3-(iodophenyl)-2(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulphonate</td>
</tr>
<tr>
<td>wt</td>
<td>weight</td>
</tr>
</tbody>
</table>
Acknowledgements

I would like to thank my supervisors Professor Shomi Bhattacharya and Dr. Peng Khaw for their help, guidance and support throughout the course of this thesis. A special thanks goes to Peng Khaw, who has taught me so much about so many things. I would also like to thank Professor Phil Luthert for providing the use of the facilities within his department. Many thanks go to the scientists who have been so kind with their time and expertise. They include: Dr. Roy Tarnuzzer and Professor Greg Schultz from the University of Florida, U.S.A.; Dr. David Baker, Dr. Peter Munro, Dr. Pete Adamson, Robin Howes, Naheed Kanuga and Karen Williams at the Institute of Ophthalmology, London. I would also like to acknowledge several of my colleagues, who are also extremely good friends, for their help and support. They include: Melville Matheson, Miranda Tighe, Lorraine Waters, Mark Wilkins and Bob Alexander at the Institute of Ophthalmology, London; and Carl Sheridan at the University of Liverpool. Many thanks also to Dr. Chee Kon for being a friend, helpful statistician and perhaps the most powerful irritant known to man.

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1. INTRODUCTION

1.1 Wound Healing: An Overview

The repair of lost or damaged adult tissue is achieved by the production of scar tissue. The healing response generally consists of a series of ordered events, some of which occur concurrently, involving several cell types, regulators of cell function and ultimately the production and remodelling of new tissue (Figure 1.1). Variations in the processes involved in this response can result in inadequate or excessive healing, both of which may result in the impairment or loss of tissue or organ function. Excessive healing in particular is a major cause of clinical morbidity, for example in atherosclerosis; restenosis following angioplasty and vein graft disease; following thermal, chemical or radiation burns; following injury or surgery, e.g., internal adhesions, intestinal blockage and keloids; and scarring due to disease, e.g., cirrhosis and scleroderma. In addition to this, the scarring response plays an extremely important role in the pathogenesis or failure of treatment of many visually disabling or blinding conditions in the world today including: cataracts; trachoma; glaucoma; burns; proliferative vitreoretinopathy (following retinal detachment or diabetes); and age-related macular degeneration.

For simplicity and discussion in the following chapter, the multistage healing process has been arbitrarily divided into early, central and late events.
Figure 1.1: Wound healing response

This figure represents a general healing response following injury. At various tissue sites, different cell types may perform the fibroblast functions outlined above. It should also be noted that several of the above events occur concurrently.
1.2 **Wound Healing: Early Events**

Immediately following tissue injury, the damage to blood vessels results in the extravasation of a variety of blood components into the wound space. Then, in combination with the response of platelets and the coagulation system, this ultimately results in the formation of a clot (cross-linked fibrin, in association with fibronectin and platelets). The formation of this clot involves several concurrently acting mechanisms including the release of locally acting mediators, an increase in vascular permeability and the influx of inflammatory cells into the wound space, all of which are described below.

1.2.1 **Role of the Blood and Clotting Systems**

In conjunction with the actions of platelets, several mediators that induce coagulation and increased vascular permeability are released following injury. Locally produced mediators that increase vascular permeability include serotonin, kinins, prostaglandins, histamine and leukotrienes (Boucek, 1984), with vascular leakage occurring for up to 72 hours. These mediators serve not only to increase vascular permeability adjacent to the wound site for clot formation to occur, but also stimulate an influx of leukocytes (primarily neutrophils). The release and actions of these locally acting mediators in increasing vascular permeability are via several mechanisms which are shown in Figures 1.2 - 1.4, and their interactions are summarised in Figure 1.5. Serotonin is derived from platelets (Boucek, 1984), while kinins such as bradykinin are derived from the actions of activated Hageman factor (factor XII) upon prekallikrein producing an active enzyme (kallikrein). Kallikrein then cleaves the bradykinin precursor kininogen, releasing active bradykinin. These processes can occur either in the plasma or within injured tissue, the mediators generated then acting directly upon the vessel wall to increase
permeability (Manjo et al. 1961; Manjo and Palade, 1961; Regoli and Barabe, 1980; Williams, 1988; Dvorak et al. 1988). Another source of similarly acting factors is the production of prostaglandins and leukotrienes. These factors are derived from the liberation of arachadonic acid via the activation of cell phospholipases and their subsequent actions on cell membrane phospholipids (Figure 1.2). Activation of the complement system also plays a role in the initiation of clotting and the influx of inflammatory cells. Liberation of the complement components C3a and C5a (Figure 1.3; adapted from Sim, 1994) directly increase vascular permeability in addition to increasing arachadonic acid metabolism, by stimulating the formation of additional permeability factors by neutrophils, and the release of histamine by resident mast cells (Robbins et al. 1984).

The clotting cascade can be divided into intrinsic and extrinsic pathways. Both of these lead to the formation of thrombin which converts fibrinogen into fibrin, and ultimately causes blood coagulation (Figure 1.4). The intrinsic pathway consists of four reaction sequences that lead to the activation of factor X, which is the enzyme required for the formation of thrombin. Major players in this clotting response are platelets which, upon exposure of sub-endothelial collagens following injury, aggregate and attach via interactions with collagen fibrils and von Willebrand factor (factor VIII) to damaged blood vessels. This is then followed by platelet degranulation (Samuelsson et al. 1978). Adhesion of platelets to exposed collagen results in the activation of Hageman factor (factor XII) which in turn then activates the clotting, kinin, complement and fibrinolytic cascades (Figure 1.5). The extrinsic pathway of the clotting cascade begins with factor VII which, upon activation by thromboplastin (tissue factor) released by endothelial cells following injury, again leads to the formation of thrombin (Figure 1.4). Following the conversion of
fibrinogen, by thrombin, to fibrin by contact with these coagulation factors (Clark et al. 1983), factor XIII then stabilises this molecule. This is then covalently cross-linked to fibronectin forming a fibrin-fibronectin matrix (Kurkinen et al. 1980; Grinnell, 1984). In addition to filling the wound space, this fibrin-fibronectin clot serves as a provisional matrix for macrophage, fibroblast and new capillary migration in addition to acting as a framework upon which collagen is later laid down (McDonald et al. 1982; Grinnell, 1984).

Platelets also appear to contribute to the initiation of inflammatory cell and fibroblast migration. As mentioned earlier, the attachment of platelets to damaged blood vessels is followed by platelet degranulation (Samuelsson et al. 1978), and a variety of factors are released including serotonin (Boucek, 1984), platelet derived growth factor (PDGF), epidermal growth factor (EGF), and transforming growth factor beta (TGFβ) (Ross et al. 1986a), which have been reported to stimulate the chemotaxis of monocytes (Deuel et al. 1982) and fibroblasts (Seppa et al. 1982).
Figure 1.2: Arachadonic acid metabolism and synthesis pathways for prostaglandins and leukotrienes

Cell Membrane Phospholipids

- Phospholipase A2

Arachadonic Acid → Lipoxygenase → 5-Hydroperoxy-eicosatraeinoic acid → Leukotrienes

- Cyclo-oxygenase

Prostaglandin Endoperoxides

<table>
<thead>
<tr>
<th>Thromboxane Synthetase</th>
<th>Thromboxane A2</th>
</tr>
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<tbody>
<tr>
<td>Prostacyclin Synthetase</td>
<td>Prostacyclin</td>
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</table>

Prostaglandins
The complement cascade generates C3a and C5a components, which are involved in increasing vascular permeability and the chemotaxis of inflammatory cells. The interaction of this cascade with coagulation, fibrinolytic and kinin systems are shown in Figure 1.5.
1.2.2 Role of Inflammatory Cells

The next stage in the healing process is the accumulation of neutrophil leukocytes at the wound site, which occurs within 24 hours post-injury (Burger et al. 1983). This involves several steps including neutrophil adherence to the endothelium, the stimulation of chemotaxis and finally the transmigration of these cells across the vascular endothelium and perivascular basement membrane. Several of the factors illustrated above in Section 1.2.1 also act as chemoattractants for neutrophils, in addition to causing increased vascular permeability. These include complement C5a, leukotrienes (Snyderman et al. 1970; Palmer et al. 1980) and TGFβ (Wahl et al. 1987). The neutrophils themselves also contribute to the increase in vascular permeability by releasing leukotrienes and platelet activating factor (Wedmore and Williams, 1981). Neutrophils are thought to primarily play a role in the destruction of bacteria introduced during tissue injury, as the depletion of circulating neutrophils has been shown to have no effect upon healing in experimental wounds (Simpson and Ross, 1972).

Within 48 hours of injury, the infiltrating neutrophils are replaced by monocytes (Issekutz et al. 1981) via the chemotactic actions of several factors including: C5a (Marder et al. 1985); fibrinopeptides (Kay et al. 1973); collagen, elastin and fibronectin fragments (Postlethwaite and Kang, 1976; Senior et al. 1980; Norris et al. 1982); TGFβ (Wahl et al. 1987) and leukotrienes (Ford-Hutchinson et al. 1980). Many of these factors are generated by the initial responses following tissue injury (see Figures 1.2 - 1.5). Monocytes then mature into tissue macrophages which, in addition to cleaning the wound by phagocytosing bacteria and cellular debris, produce a plethora of growth factors which influence the control of the following healing process (Knighton et al. 1984; Riches, 1988; Knighton and Fiegel, 1989). This is supported by the fact that depletion of circulating
monocytes and tissue macrophages has been shown to result in a reduction of wound fibrosis (Leibovich and Ross, 1975). Growth factors produced by macrophages include members of the TGFβ, FGF, PDGF and EGF families (Baird et al. 1985; Shimokado et al. 1985; Assoain et al. 1987; Madtes et al. 1988), which have a variety of effects on cells involved in the wound healing process including endothelial cells and fibroblasts (see Section 1.5 for a more in-depth treatise). Following this initial response, inflammation is then resolved following granulation tissue formation (see below). This is thought to include the dissipation of mediators; return of normal vascular permeability; and removal of fluids, proteins, debris (bacterial and cellular), neutrophils and finally macrophages (Haslett and Henson, 1988). The next overlapping stage of the healing process to occur is the formation of granulation tissue.
**Figure 1.4**: Coagulation pathways initiated by tissue injury

Fibrillar Collagen + Factor XII

+ Prekallikrein + Kininogen

Factor XII (activated)

Kininogen

Factor XI → Factor XI (activated)

Factor IX → Factor IX (activated) + Calcium + Platelet + Factor VIII (activated)

Lipid

Factor VIII

Tissue Thromboplastin + Factor VII

Factor VII (activated)

Factor X → Factor X (activated) + Calcium + Platelet + Factor V (activated)

Lipid

Factor V

Prothrombin → Thrombin

Fibrinogen → Fibrin

The intrinsic (■) and extrinsic (□) clotting pathways lead to the generation of thrombin and ultimately a fibrin clot. Interaction of these pathways with the complement, fibrinolytic and kinin systems are shown in Figure 1.5
Figure 1.5: Interactions between coagulation, fibrinolytic, complement and kinin systems following tissue injury.
1.3 **Wound Healing: Central Events**

The formation of granulation tissue includes the simultaneous accumulation of macrophages, described above in Section 1.2, the migration and proliferation of fibroblasts, extracellular matrix (ECM) contraction and the ingrowth of new blood vessels (angiogenesis) at the wound site (Hunt, 1980). Many of these cellular functions are regulated by a number of chemotactic and growth factors, which are highlighted below and in Section 1.5.

1.3.1 **Roles of the Fibroblast**

Fibroblasts normally exist in the perivascular connective tissue as quiescent undifferentiated cells; fibrocytes. Following the influx of inflammatory cells into the wound a number of biologically active substances, including growth factors, result in the activation of quiescent fibrocytes into active fibroblasts. The fibroblast is the key player in the wound healing process, carrying out a number of crucial functions including: migration; wound contraction; proliferation; synthesis of new ECM and remodelling of this matrix. As such, much research has concentrated on understanding the mechanisms underlying the regulation of fibroblast function. In a few specialised parts of the body other cells carry out this pivotal part of the healing process, e.g., retinal pigmented epithelial cells in the retina, glia in the central nervous system and smooth muscle cells in the vasculature. The variety of healing functions carried out by fibroblasts, their control and underlying mechanisms are given below.
1.3.1.1 Migration

The movement of fibroblasts into the wound site occurs within 48-72 hours post-injury. Although the exact mechanisms of this process are currently unclear, several key components have been identified experimentally. A number of in vitro assay systems have been used to study cellular migration including: Boyden migration chambers (Boyden, 1962); two compartment Boyden chambers (Zigmond and Hirsch, 1973); orientation chambers (Zigmond, 1977); under agarose (Nelson et al. 1978); and modified forty-eight microwell two tiered blindwell Boyden migration chambers. Several in vitro studies have suggested that the movement of fibroblasts to the wound site in vivo may be mediated by various polypeptides including: inflammatory cell products (Postlethwaite et al. 1976; Sobel and Gallin, 1979; Postlethwaite and Kang, 1980); complement (Postlethwaite et al. 1979); PDGF (Seppa et al. 1982); TGFβ (Postlethwaite et al. 1987); and ECM components including collagen (Postlethwaite et al. 1978), elastin (Senior et al. 1984), fibronectin (Joseph et al. 1987); and fibrinogen derivatives (Senior et al. 1986). These polypeptides (termed chemoattractants) are regarded as stimulating migration through the process of chemotaxis. Chemotaxis has been defined as the directed migration of cells in response to a concentration gradient of a soluble chemoattractant (McCarthy et al. 1988) and is regarded as the major mechanism by which cellular migration is controlled. In addition to chemotaxis, the movement of cells independently of the chemoattractant gradient (termed chemokinesis; Zigmond and Hirsch, 1973); haptotaxis (the movement of cells via a substratum bound gradient of a particular matrix constituent; Harris, 1973); and contact guidance (the tendency of cells to align and move along discontinuities in the ECM (Weiss, 1945; Weiss, 1958); may also contribute to the migration of cells into the wound site. The movement of cells appears to involve several sequential steps. These include the
extension of cytoplasmic processes (lamellipodia); the attachment of the cell to its substratum; dis/assembly and contraction of the actin cytoskeleton; and as the cell moves forwards, deattachment of cell-substratum adhesions. One of the initial mechanisms involved in cell movement is the production of cell cytoplasmic processes (lamellipodia). Lamellipodia are structures filled with a dense meshwork of actin filaments, and their chief function is to advance the leading edge of the cell forwards. These lamellipodia can rearrange very rapidly via several mechanisms involving actin de/polymerisation (Theriot, 1994).

The adhesion of cells to their substratum is mediated by a family of cell surface expressed receptors called integrins. The integrins are a family of heterodimeric glycoproteins which consist of two subunits; α and β chains. Examples of these glycoproteins include: α<sub>1</sub>β<sub>1</sub>, α<sub>2</sub>β<sub>1</sub>, α<sub>3</sub>β<sub>1</sub>, α<sub>4</sub>β<sub>1</sub>, α<sub>5</sub>β<sub>1</sub>, α<sub>6</sub>β<sub>1</sub> and α<sub>i</sub>β<sub>j</sub>, which are receptors for ECM molecules including collagens, fibronectin, vitronectin and thrombospondin (Hynes, 1992). Integrins also provide a physical link to the cytoskeleton and play a role in the transduction of extracellular signals (Langholz et al. 1995; Hotchin and Hall, 1995). In the presence of ECM molecules and extracellular growth factors, integrins can become activated forming clusters and focal adhesion complexes. This results in the generation of second messengers within the cell via several pathways involving mitogen activated protein (MAP) kinases, which ultimately lead to changes in gene expression (Hotchin and Hall, 1995; Stuiver and O'Toole, 1995; Richardson and Parsons, 1995). The formation of focal adhesion complexes and the subsequent transduction of signals within the cell, have been shown to be dependent upon intracellular signalling cascades involving enzymes such as phosphoinositol 3-kinase (PI3-K; Otsu et al. 1991), and its up and downstream regulators including growth factor receptors and cytoplasmic tyrosine kinases, the Rho family of
proteins (Hotchin and Hall, 1995), protein kinase C and focal adhesion kinase (FAK). The interactions of these pathways are shown schematically in Figure 1.6 (adapted from Matsumoto et al. 1995).

Although several of the mechanisms described above have been shown to be involved in cellular migration in in vitro models, the exact mechanisms involved in the migration of cells in vivo may be different. This may be due to the fact that cells in vivo are either situated upon ECM components or are surrounded by this matrix. Several in vitro studies, utilising pseudo-in vivo models, have demonstrated that the migration/invasion of a number of cell types across or through ECM components is dependent upon matrix metalloproteinase (MMP) activity (Pauly et al. 1994; Taraboletti et al. 1995; Leppert et al. 1995), as is angiogenesis (Galardy et al. 1994; Taraboletti et al. 1995; also see Section 1.4.1.5).
Figure 1.6: Potential signalling pathways involved in growth factor induced cell motility

**MOTILITY / INVASION**

↑ Growth factor / growth factor receptor
↓ Integrin

![Diagram of signalling pathways]

**IMMOBILISATION**

↓ Growth factor / growth factor receptor
↑ Integrin

**KEY**

- Growth factor
- Growth factor receptor
- Integrin

FAK = focal adhesion kinase
R TyrK = receptor tyrosine kinase
MAPK = mitogen activated protein kinase
PI3-K = phosphoinositide 3-kinase

(Adapted from Matsumoto et al, 1995)
1.3.1.2 ECM Contraction

The contraction of collagen containing tissues is fundamental not only to the wound healing process (Grinnell, 1994) but also tissue morphogenesis (Stopak and Harris, 1982; Lewis, 1984) and embryonic development (Brenner et al. 1989). ECM contraction was originally thought to be due to the actions of specialised cells exhibiting smooth muscle cell characteristics; myofibroblasts (Gabbiani et al. 1971; Majno et al. 1971). These cells exhibited several characteristics including the expression of α-smooth muscle actin, convoluted nuclei and a spindle shaped morphology. It was postulated that these cells contracted as a syncitium in a similar fashion to muscle cells, ultimately resulting in wound closure. However, the fibroblast-mediated contraction of the ECM is now regarded as resulting from the tractional forces exerted by migrating fibroblasts upon their substratum (Harris et al. 1981; Ehrlich and Rajaratnam, 1990).

The behaviour of cells in a collagen solution *in vitro*, which forms a three-dimensional network (Wood, 1964), was originally compared to that of cells on a plastic substratum (Elsdale and Bard, 1972). Subsequently, many groups have used an *in vitro* model of collagen contraction consisting of fibroblasts entrapped within a three-dimensional collagen matrix, which was originally described by Bell *et al* (1979). The cells within this matrix reorganise and subsequently contract the matrix over a period of several days, the morphological characteristics of these cells resembling those seen *in vivo* (Tomasek and Hay, 1984). Studies using this model consisting of fibroblasts seeded within or upon three-dimensional collagen matrices (Bell *et al*. 1979; Schor, 1980; Guidry and Grinnell, 1985), have demonstrated that contraction is dependent upon a variety of factors including: collagen concentration and cell number (Bell *et al*. 1979; Allen and Schor, 1983; Buttle and Ehrlich, 1983); an intact actin cytoskeleton (Bell *et al*. 1979; Guidry and
Grinnell, 1985); attachment of cells to their surrounding matrix (Schiro et al. 1991; Klein et al. 1991); and protein synthesis (Guidry and Grinnell, 1985). In addition to this, the contraction of collagen has been shown to be stimulated by a variety of growth factors (see Section 1.5) and to involve intracellular signalling cascades (Guidry, 1993).

Although much research upon the process of collagen contraction has been carried out, the exact mechanisms underlying this process are currently unclear. Such mechanisms include the elucidation of how fibroblasts within a three-dimensional matrix migrate, are signalled to start and stop contraction, and how these cells respond to changes in biophysical forces within granulation tissue. One possible way of studying the cellular and molecular behaviour of fibroblasts within ECM is by using a novel system; the Culture Force Monitor (CFM). The original prototype of the CFM allowed accurate, sensitive and reproducible measurement of the contractile forces generated by cells within a collagen matrix (Eastwood et al. 1994). Other instruments have now been developed by Eastwood et al to investigate the effects of biophysical forces on cellular behaviour in ECM (tension CFM), and the formation of adhesions between tissue interfaces in vitro (adhesion CFM; Cacou et al. 1996).

1.3.1.3 Proliferation

Following the migration of fibroblasts to the wound site and the resultant contraction of the ECM, they then proliferate reaching maximal numbers within one to two weeks post injury (Ross and Odland, 1968). The proliferation of fibroblasts at the wound site is important in order for the cell number to be sufficient to allow adequate healing. As for fibroblast migration and ECM contraction, the proliferation of these cells is stimulated by a number of growth factors (see Section 1.5). However, these growth
factors also have inhibitory as well as stimulatory effects on cellular functions related to wound healing, depending upon the cell type studied (see Table I). The quantitation of proliferation in response to a variety of stimuli has been achieved using several methods including \(^3\)H-thymidine assays (Freshney, 1987), counted cell number e.g., haemocytometer, Coulter counter, and colourimetric assays e.g., MTT and WST assays based upon cellular metabolic activity (Mosmann, 1983; Kawase et al. 1995), and the methylene blue assay based upon dye binding to the cell monolayer (Finlay et al. 1984).

### 1.3.2 Role of Endothelial Cells

Neovascularisation of the wound occurs simultaneously with the migration into and the proliferation of fibroblasts at the wound site. The process of formation of capillary beds via the influx of microvessel buds from the surrounding tissue vasculature (angiogenesis) is a central component of the wound healing process. Angiogenesis is a complex process and is thought to involve several stages including migration, growth and capillary tube formation. As for the other stages of wound healing, angiogenesis is regulated by several factors including chemical mediators (also see section 1.5). Interestingly recent work has suggested a role for the MMPs in angiogenesis (Galardy et al. 1994).
13.3 Role of Epithelial Cells.

Re-epithelialisation is an important factor in the closure of wounds. Two different mechanisms have been reported to be involved in the re-epithelialisation of wounds. Studies by Martin and Lewis (1992) and Bement et al (1993), have suggested a purse string closure mechanism involving the formation of an actin cable in foetal epithelial healing and gastrointestinal epithelial healing. In addition, it is also thought that basal epithelial cells migrate directionally across the wound defect pulling the upper layers of the epidermis passively along, followed by proliferation and finally deposition of a new basement membrane and restratification of the epidermis (Mackenzie and Fusenig, 1983). Recently, this migration of epithelial cells across a wound defect have been shown to involve the action of MMPs (Woodley et al. 1986; Saarialho et al. 1993; Iwasaki et al. 1994; Saarialho-Kere et al. 1995). Once re-epithelialisation has occurred, the remodelling and maturation phases of the healing process begin (see Section 1.4)

1.4 Wound Healing: Late Events

1.4.1 Extracellular Matrix Production and Remodelling

The reformation of the ECM begins simultaneously with the formation of granulation tissue by inflammatory cells, fibroblasts and endothelial cells. Initially high levels of fibronectin and hyaluronic acid are deposited at the wound site, disappearing as the matrix matures over several weeks with increasing wound collagen and proteoglycan content. In addition to repairing the ECM damage, the production of collagen types I and III as well as fibronectin and fibrin provides support for epithelial cell migration (Clark et al. 1982; Woodley et al. 1985) and thus re-epithelialisation of the wound. The production
of new ECM at the wound site involves several cell types involved in the healing process. For example, fibronectin is derived from macrophages, endothelial cells, fibroblasts and some epithelial cells (Oh et al. 1981; Clark et al. 1982), while proteoglycans have been shown to be produced by fibroblasts, smooth muscle and endothelial cells (Paulsson et al. 1986; Lane et al. 1986). However it is the fibroblast which is the major producer of new ECM, these cells secreting a number of ECM molecules including fibronectin, glycosaminoglycans and collagen types I and III (Barnes et al. 1976; Williams et al. 1984). The production and roles of these molecules in the healing process are highlighted below.

1.4.1.1 ECM Production: Fibronectin

Fibronectin is a dimeric glycoprotein (M, 440-500 kD) that has been found in two forms. The first is a soluble form that is present in plasma, and the second is an insoluble form which is assembled into a matrix around secreting cells such as fibroblasts and endothelial cells (McDonald, 1988). Fibronectin has at least two functional regions, one that allows interactions with other ECM components and one that regulates cellular adhesion/migration. Fibronectin is a particularly adhesive molecule, binding to several other components of the ECM including fibrin, collagens and glycosaminoglycans as well as cell-surface receptors (integrins; see Section 1.3.1.1) on a variety of cell types including fibroblasts (McDonald, 1988). The interaction of fibronectin with cells is important as it has been shown to play a role in a number of processes including adhesion, migration, growth and differentiation (Ruoslahti, 1988); the promotion and assembly of the ECM, as well as the control of gene induction, all of which are important in the healing process.
1.4.1.2 ECM Production: Proteoglycans and Glycoproteins

Proteoglycans consist of a core protein to which polysaccharides (glycosaminoglycans) are covalently linked. Members of this group of molecules include: chondroitin sulphate; heparan sulphate; and keratan sulphate. Although these molecules have not been well studied, roles for them in morphogenesis and wound healing have been suggested (Bernfield and Banjee, 1972; Ausprunk et al. 1981).

Members of the group of molecules known as the glycoproteins include: fibronectin (see Section 1.4.1.1); thrombospondin; and vitronectin. Thrombospondin has been shown to be produced by a number of cell types involved in the healing process including: endothelial cells; some epithelial cells; macrophages; and fibroblasts (Lawler, 1986; Jaffe et al. 1983). Thrombospondin has been implicated in both assembly of the ECM and the migration of cells following injury (Raugi et al. 1987; Lawler, 1986). The glycoprotein vitronectin is present at high concentrations in the plasma and is also produced by several cell types including platelets and monocytes/macrophages (Preissner, 1991). This molecule is known to have binding sites for several molecules including: integrins; other ECM components; and complement components suggesting a role for this molecule in tissue repair (Preissner, 1991).

1.4.1.3 ECM Production: Collagens

Eighteen distinct members of the collagen family have been described (Kielty et al. 1993). Each collagen molecule consists of a triple helix of three polypeptide \( \alpha \) chains either as heterodimers in the case of type I collagen \((\alpha_1(I)_2 \alpha_2(I))\), or homodimers for example in type III collagen \((\alpha_1(III)_3)\) and have molecular weights ranging from 280-530kD. Following translation, the collagen polypeptides are modified.
via hydroxylation of proline and lysine residues in addition to the glycosylation of hydroxylysine residues. The pro-\(\alpha\) chains are then arranged intracellularly into a triple helical conformation, in conjunction with interchain disulphide bonding. These molecules are then secreted into the extracellular space forming the collagen precursor tropocollagen, via the loss of \(\text{NH}_2\) and \(\text{COOH}\) domains (Gabbiani and Montandon, 1977). This is followed by tropocollagen covalent crosslinkage by the enzyme lysyl oxidase, the degree of cross-linking reflecting the strength of the healed tissue (Chvapil and Koopmann, 1984).

Collagen synthesis during wound healing is carried out by the fibroblast. Collagen is the major component of the wound matrix and as such two members of this large family of proteins, collagen types I and III, are highlighted here since the roles of these in wound healing is best defined. The first collagen to be laid down at the wound site is collagen type III (Gabbiani et al. 1976; Guber and Ross, 1978) within 10-72 hours post injury (Clore et al. 1979). Collagen type III production then peaks maximally at between days 5 to 7. The new collagen type III is laid down in close association with pre-existing fibrin networks, and subsequently stabilised by mucopolysaccharides (Ross and Odland, 1968). Following the regression of both fibroblasts and endothelial cells, collagen type III is replaced by collagen type I (Dvorak, 1986). A number of growth factors have been shown to stimulate ECM synthesis including PDGF (Grotendorst et al. 1985), TGF\(\beta\) (Sporn et al. 1983; Ignotz and Massague, 1986), EGF, insulin-like growth factor I (IGF-I) and basic fibroblast growth factor (bFGF; also see Table 1.2).
1.4.1.4 ECM Remodelling: Plasminogen Activators (PAs)

Several degradative enzymes play crucial roles in the remodelling of the existing and newly synthesised ECM throughout the healing process. For discussion, these enzymes have been divided into two groups: plasminogen activators (PAs) and matrix metalloproteinases (MMPs). The PAs include urokinase (u-PA) and tissue-type (t-PA) and are capable of activating plasminogen to plasmin, which has been shown to be involved in the degradation of fibrin clots (Robbins et al. 1981). u-PA has been shown to be produced by inflammatory cells (Gordon et al. 1974; Vassalli et al. 1984) and it is thought that in conjunction with t-PA derived from vessel disruption, leads to the degradation of the fibrin clot. In addition to this, plasmin has been shown to degrade fibronectin (Werb et al. 1980) and to activate the latent forms of MMPs (Werb et al. 1977). Plasminogen activators have also been implicated in the migration of fibroblasts and endothelial cells, although the exact mechanisms of this process are unclear, as well as the proliferative process during arterial repair (Wysocki et al. 1996). The interactions of PAs during tissue remodelling are summarised in Figure 1.7.

1.4.1.5 ECM Remodelling: Matrix Metalloproteinases (MMPs)

The other major group of ECM degrading enzymes are the MMPs, which are involved in the degradation of collagens (types I-V and VII-XI), gelatin (denatured collagen), fibronectin, laminin, elastin and proteoglycan core protein (Woessner, 1991; Matrisian, 1992; Birkedal-Hansen et al. 1993; Birkedal-Hansen, 1995). There are several members of the MMP family which share a number of structural and functional features including a Zn$^{2+}$ binding site, and may be regarded as derivatives (formed by deletion or addition of domains) of the modular structure of collagenases and stromelysins. Members
of this family include MMP 1 (collagenase), MMP 2 (72kD gelatinase), MMP 3 (stromelysin), MMP 7 (PUMP-1, putative metalloproteinase-1), MMP 8 (neutrophil collagenase), MMP 9 (92kD gelatinase), MMP 10 (stromelysin-2) and MMP 11 (stromelysin-3). Individual MMPs tend to cleave specific ECM substrates, although there is some overlap in the substrate specificity of these enzymes. These enzymes are initially secreted in an inactive or proform, and are subsequently activated extracellularly (see Figure 1.7). MMPs have been reported to be produced by a variety of cells involved in the wound healing process including neutrophils, macrophages, fibroblasts, endothelial cells, keratinocytes and vascular smooth muscle cells (Birkedal-Hansen et al. 1993; Pauly et al. 1994), their production being regulated by a number of external stimuli including growth factors. Another characteristic of these enzymes is that their activity is regulated by the tissue inhibitors of matrix metalloproteinases (TIMPs; see Figure 1.7), of which three forms have currently been reported (TIMP 1, 2 and 3). The degree of degradation of the ECM is dependent upon the balance between the ratio of MMPs : TIMPs, a relative increase in MMPs or a decrease in TIMPs resulting in an overall increase in degradation and vice versa. An example of the importance of the balance of these two systems in ECM turnover has been highlighted in restenosis, where Tyagi et al (1995) reported an increase in ECM production and a decrease in MMP activity compared to normal artery. Studies performed by Newby et al (1994) have also suggested that the MMPs may be involved in the proliferation and outgrowth of vascular smooth muscle cells in atherosclerosis.

In addition to these secreted MMPs, two members of a novel subclass of the MMP family, membrane type-matrix metalloproteinases (MT-MMPs) 1 and 2, have recently been reported (Sato et al. 1994; Takino et al. 1995). Although the exact roles of MT-MMPs in wound healing are currently unclear, it is thought that they may play a role in MMP
activation at the cell surface (Takino et al. 1995; Lewalle et al. 1995; Strongin et al. 1995) see Figure 1.7).

In addition to their roles in ECM remodelling, the MMPs have also been implicated in other stages of the wound healing response including the migration of cells across or through the ECM during inflammation, angiogenesis, re-epithelialisation and cellular influx into the wound site (see Section 1.3.1.1), and the release of ECM-bound growth factors. As such, the MMPs appear to be pivotal players in the healing process regardless of cell type or tissue site.
The degree of extracellular matrix turnover is dependent upon the balance between the ratios of stimulatory (e.g., MMPs) and inhibitory (e.g., TIMPs) factors.
1.5 Role of Growth Factors and Their Receptors in Wound Healing

Several growth factors have been implicated in the wound healing process including members of the EGF, TGFβ, IGF, PDGF and FGF families. These growth factors are thought to play many key roles not only in the initiation but also in sustaining the wound healing process, the properties and molecular biology of these factors being the subject of several reviews (Clark and Henson, 1988; Bennett and Schultz, 1993a; Bennett and Schultz, 1993b). The roles of growth factors include the stimulation or inhibition of several cellular functions including proliferation, migration, ECM contraction and ECM synthesis. The major sources of growth factors at the wound site, and the effects of these growth factors on cell function regulation relating to wound healing are summarised in Tables 1.1 and 1.2 respectively. Although the effects and roles of individual growth factors have been extensively studied in vitro, their interactions and roles in vivo during the wound healing process are obviously extremely complex and as yet are incompletely elucidated. Members of the different growth factor families are discussed briefly below with selected aspects of their discovery, modes of action, receptors and importance in the wound healing process, being highlighted.
Table 1.1: Major sources of growth factors at the wound site

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDGF</td>
<td>Platelets, macrophages, epithelial cells,</td>
</tr>
<tr>
<td></td>
<td>endothelial cells, smooth muscle cells</td>
</tr>
<tr>
<td>TGFβ</td>
<td>Platelets, macrophages, activated</td>
</tr>
<tr>
<td></td>
<td>T-lymphocytes</td>
</tr>
<tr>
<td>EGF / TGFα</td>
<td>Platelets, macrophages, epithelial cells,</td>
</tr>
<tr>
<td></td>
<td>eosinophils (TGF-α)</td>
</tr>
<tr>
<td>IGF-I</td>
<td>Plasma, epithelial cells, endothelial cells,</td>
</tr>
<tr>
<td></td>
<td>fibroblasts, smooth muscle cells</td>
</tr>
<tr>
<td>bFGF</td>
<td>Macrophages, endothelial cells</td>
</tr>
</tbody>
</table>

(Adapted from Bennett and Schultz, 1993a)
Table 1.2: Effects of growth factors on connective tissue repair

<table>
<thead>
<tr>
<th>Growth Factor</th>
<th>Molecular Weight (kD)</th>
<th>Report Effects on Cellular Functions Related to Wound Healing</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Fibroblasts</td>
</tr>
<tr>
<td>EGF 6</td>
<td></td>
<td>Stimulates migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ECM contraction</td>
</tr>
<tr>
<td>TGFα 5-20</td>
<td></td>
<td>see effects of EGF above</td>
</tr>
<tr>
<td>TGFβ 25</td>
<td></td>
<td>Stimulates migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ECM contraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC synthesis</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IGF-1 7.5</td>
<td></td>
<td>Stimulates migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ECM contraction</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EC synthesis</td>
</tr>
<tr>
<td>PDGF 28-35</td>
<td></td>
<td>Stimulates migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>proliferation</td>
</tr>
<tr>
<td>FGF (acidic and basic) 16-18</td>
<td></td>
<td>Stimulates migration</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ECM contraction</td>
</tr>
</tbody>
</table>

(Adapted from Bennett and Schultz, 1993a)
1.5.1 Growth Factors and the Cell Cycle

The process of proliferation in eukaryotic cells involves a well-ordered series of biochemical and genetic events which ultimately result in cell division. This process is termed the cell cycle and can be divided into five phases; G0, G1, S, G2 and M phases. The progression of cells through the cell cycle is controlled by the formation, activation and subsequent inactivation of a series of cyclin-dependent kinases and their regulatory subunits, the cyclins (Elledge and Harper, 1994; Peters, 1994). The G0/G1 phase is characterised by cells which are either quiescent (G0) or exhibit an increase in biosynthetic activities (G1), including increased RNA and protein synthesis. Several factors including cell density, availability of nutrients and the presence of growth factors can influence the decision of a cell to progress through a restriction point (R) in mid-late G1 onto DNA synthesis, or to enter quiescence (G0) (Sorrentino, 1989). During the S phase, DNA synthesis occurs followed by a gap period (G2) before mitosis (M) starts (Pardee, 1989).

Smith and Stiles (Smith and Stiles, 1981) proposed a simplified scheme whereby growth factors can be divided into two groups based upon their actions at different phases of their cell cycle. The first group, termed competence factors, exert their actions upon genes controlling early G0/G1 events and make the cell responsive to the second group of factors, the progression factors, which regulate processes later on in the G1 phase. Most growth factors either exhibit competence or progression activity, not usually both.

1.5.2 Growth Factor Secretion

The mechanisms by which growth factors are secreted are 'type-dependant' (Lawrence et al. 1985; Ignotz et al. 1986a; D'Amore, 1990; Cross and Dexter, 1991) and may involve: classical secretory pathways via translocation through the endoplasmic
reticulum and then in vesicles through the Golgi to the plasma membrane, e.g., PDGF, IGFs; novel secretory pathways for growth factors lacking the six or seven hydrophobic amino acid signal sequence required for translocation across the endoplasmic reticulum, e.g., FGFs (Meusch et al. 1990); secretion, by classical pathways, of a high molecular weight or membrane-bound complex which is subsequently rendered active by proteases, e.g., TGFβ, EGF / TGFα.

1.5.3 Mechanisms of Action of Growth Factors

Unlike hormones which act via endocrine mechanisms, growth factors exert their effects mainly by: paracrine mechanisms whereby the effects of a growth factor are elicited by receptors upon cells lying in close proximity to the secreting cell, e.g., most growth factors (Aaronson, 1991); juxtacrine mechanisms whereby a high molecular weight membrane-bound precursor form of the growth factors stimulates receptors on neighbouring cells i.e., this mechanism does not involve the transfer of molecules by diffusion, e.g., EGF / TGFα (Wong et al. 1989); or external autocrine mechanisms where the growth factor exerts its effects upon the secreting cell itself or upon neighbouring cells of the same type (Halaban et al. 1988; Browder et al. 1989), e.g., TGFβ, bFGF ; or internal autocrine mechanisms which involve the intracellular binding of the growth factor to its receptor with subsequent receptor activation (Keating and Williams, 1988; Browder et al. 1989), e.g., PDGF.

In general, the process where by the interaction of a growth factor with its specific receptor produces a cellular response involves a series of molecular mechanisms. Processes include an increase in the tyrosine kinase activity associated with the receptor resulting in phosphorylation of phospholipase C. This phosphorylated enzyme cleaves
phosphatidylinositol 4,5-bisphosphate to inositol 1, 4, 5-triphosphate and 1, 2-diacylglycerol. Inositol 1, 4, 5-triphosphate then diffuses through the cytosol to the endoplasmic reticulum where it causes a calcium efflux into the cytosol. 1, 2-diacylglycerol remains associated with the membrane where in association with calcium, it activates protein kinase C. Activated protein kinase C then phosphorylates several other proteins altering their activities (Darnell et al. 1990), including MAP kinases which then effect several nuclear proteins. The nuclear proteins activated include the nuclear transcription factors \(c\text{-}myc\) and \(c\text{-}fos\), which leads to a cellular response (i.e., cell division).

### 1.5.4 Fibroblast Growth Factors (FGFs)

Fibroblast growth factor activity was initially reported in brain-tissue extracts (Trowell et al. 1939) and subsequently 'rediscovered' some thirty five years later (Armelin, 1973). A component of these extracts was partially purified and found to be acid and heat labile with a basic isoelectric point (bFGF; Gospodarowicz, 1974b). A second distinct component was detected in brain-tissue extracts (Thomas et al. 1980), and was termed acidic fibroblast growth factor (aFGF) in contrast to its basic counterpart. These two growth factors were purified to homogeneity (Thomas et al. 1984; Bohlen et al. 1984) and found to have molecular weights of 16 kD (aFGF) and 18 kD (bFGF) by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis, as well as to elute from heparin Sepharose at 1M and 2M NaCl respectively (Shing et al. 1984; Lobb et al. 1986). The amino acid sequences of aFGF (Gimenez-Gallego et al. 1985; Gimenez-Gallego et al. 1986a) and bFGF (Esch et al. 1985) were subsequently determined to be 53% homologous, indicating that aFGF and bFGF were structurally related, although they are located on different chromosomes. \textit{In vitro}, FGFs have been reported to have mitogenic
effects upon fibroblasts and endothelial cells (Gospodarowicz et al. 1986b; Gospodarowicz et al. 1986c). Similar effects are observed *in vivo* with FGFs stimulating the mitogenesis of fibroblasts (Davidson et al. 1985) and angiogenesis (Gospodarowicz et al. 1986a). In most biological systems, bFGF tends to be 10-100 times more potent a mitogen than aFGF (Gospodarowicz et al. 1986a). Unlike bFGF, the activity of aFGF is potentiated by heparin (Gimenez-Gallego et al. 1986b) which probably induces a more active conformation of the aFGF molecule (Barzu et al. 1989). The mechanism of action of FGF release is at present unknown as although the FGFs are found in high concentrations extracellularly, bound to ECM components such as heparan sulphate, these growth factors lack signal sequences which directs proteins into the classical secretory pathway (Meusch et al. 1990).

Models for secretion for these growth factors have included release from dead or dying cells, although release from viable, apparently uninjured cells has been demonstrated (Mignati and Rifkin, 1991). It has however, been proposed that FGFs may be released by cell leakage, sublethal cell injury, a novel secretion pathway or any combination of these (D'Amore, 1990).

Receptors for FGFs have been demonstrated on a variety of cell types including endothelial cells (Friesel et al. 1986) and fibroblasts (Olwin and Hauschka, 1986). The FGF receptors are membrane-bound proteins consisting of a single polypeptide chain possessing a molecular weight range of 110 - 150 kD and are present as high or low affinity binding sites (Moscatelli, 1987; Moscatelli, 1988). Several intracellular pathways have been implicated in FGF signal transduction including the activation of guanylate cyclase (Tsuda et al. 1986), phospholipase breakdown of phosphatidylinositides (Tsuda et al.
1985) and activation of protein kinase C, as well as tyrosine kinase activity associated with the receptor itself (Huang and Huang, 1985).

In vitro, bFGF has been shown to stimulate several cellular functions involved in the healing process. These include the stimulation of: fibroblast and endothelial cell migration (Grant et al. 1992; Khaw et al. 1994b); ECM contraction by fibroblasts (Assouline et al. 1992; Khaw et al. 1994b); fibroblast proliferation (Gospodarowicz, 1974a); fibroblast ECM production (Khaw et al. 1994b) and fibroblast MMP production (Buckley-Sturrock et al. 1989). Cells reported to produce this growth factor at the wound site include fibroblasts and endothelial cells (Bennett and Schultz, 1993b). The involvement of bFGF in the healing process in vivo has also been shown, with this factor stimulating angiogenesis; increasing the tensile strength of scar tissue following surgical incisions (McGee et al. 1988); enhancing the healing response in the cornea (Petroutsos et al. 1984) and gastrointestinal tract (Folkman et al. 1991); and stimulating nerve regeneration (Danielsen et al. 1988; Aebischer et al. 1989; Cordeiro et al. 1989).

1.5.5 Platelet-Derived Growth Factors (PDGF)

Platelet-derived growth factor (PDGF), was discovered to be the major mitogenic component present in the whole blood for various cultured mesenchymal cells, including arterial smooth muscle cells (Ross et al. 1974). PDGF was initially purified from human platelets (Antoniades, 1981), and was shown to have a M, ranging from 28 to 35 kD. The PDGF molecule was found to consist of two distinct polypeptide chains termed A and B (Antoniades and Hunkapiller, 1983) which shared sequence homology. Three isoforms of the PDGF molecule have been isolated from platelets, the majority of PDGF released being present as the heterodimeric PDGF_{AB} form (Hammacher et al. 1988), the remainder
consisting of the PDGF_{bb} (Hammacher et al. 1988) and the PDGF_{aa} homodimeric forms (Hart et al. 1989). PDGF has been shown to be mitogenic both for fibroblasts and small vessel endothelial cells (Rutherford and Ross, 1976; Bar et al. 1989).

Initial observations indicated that the PDGF receptor existed as a single transmembrane glycoprotein with an extracellular ligand-binding domain, and an intracellular domain which exhibited tyrosine kinase activity. These observations followed the discovery of a PDGF receptor with a M, of approximately 160 kDa (Glenn et al. 1982). Subsequent studies however, showed that there were at least two different PDGF receptors: one which binds all three PDGF isoforms - the A/B receptor, and a form which binds the PDGF_{bb} homodimer - the BB receptor (Hart et al. 1988). The cloning of the complementary deoxyribonucleic acid (cDNA) for the respective subunits of the PDGF receptor α (Matsui et al. 1989) and β (Claesson-Welsh et al. 1988) subunits, led to the proposal that the high affinity receptor for the PDGF isoforms is a dimer consisting of either α subunits (binds A or B chains), or β subunits which bind the B chains only (Seifert et al. 1988).

Upon ligand binding to the PDGF receptor, autophosphorylation occurs (Ek et al. 1982) followed by increased phosphatidylinositol turnover (Habernicht et al. 1985), which in turn leads to a cellular response. After binding the ligand is internalised and degraded (Bowen-Pope and Ross, 1982), indicating that the receptor is also internalised and degraded rather than being recycled.

PDGF is produced by several cell types at the wound site including platelets, monocytes, endothelial cells and fibroblasts (Ross et al. 1986b). This growth factor has several stimulatory effects on the wound healing behaviour of cells including stimulation of neutrophil, monocyte and fibroblast migration (Deuel and Huang, 1984); stimulation of
fibroblast ECM contraction and proliferation (Gullberg et al. 1990; Assouline et al. 1992); and stimulation of fibroblast ECM and MMP production (Narayanap and Page, 1983; Bauer et al. 1985). PDGF has been shown to stimulate the healing of chronic ulcers in vivo (Robson et al. 1992), but only increases the rate of healing or an increase in wound DNA content in conjunction with IGF-I or bFGF in vivo (Lynch et al. 1987).

1.5.6 Insulin-Like Growth Factors (IGFs)

IGF-I and IGF-II are both single polypeptide chains consisting of 70 and 67 amino acids respectively (Humbel, 1984). Both of the insulin-like growth factors are derived from higher molecular weight precursor forms. Whereas there are two IGF-I precursor forms, there is only one IGF-II precursor which has a $M_r$ of 21 kD as determined by cDNA sequencing. The first IGF-I precursor form (termed prepro-IGF-Ia) has a $M_r$ of 11.7kD, the second having a $M_r$ of 21 kD and is termed prepro-IGF-Ib (Jansen et al. 1983). These two different precursor forms are thought to arise by alternate RNA splicing. IGF-I has been shown to be chemotactic for bovine aortic and human retinal capillary endothelial cells (Grant et al. 1993). However studies in which insulin and IGFs have no effect on the growth of endothelial cells cultured from large blood vessels, such as bovine aorta and pulmonary artery, have also been reported (Bar et al. 1988).

There are two types of IGF receptor, one has a high affinity for IGF-I but weak affinities for IGF-II and insulin, and one binds IGF-II with high affinity while IGF-I is bound with low affinity and insulin not at all (Rechler et al. 1980). These receptors are termed type I and type II receptors respectively. The type I receptor consists of $\alpha$ subunits (which have a $M_r$ of 130 kD) and $\beta$ subunits (which have a $M_r$ of 95 kD), in $\alpha_2\beta_2$ heterodimeric complex. Phosphorylation of the type I receptor is ligand-binding
dependant, phosphorylation being confined to the tyrosine residues of the β subunits ( Jacobs et al. 1983; Yu et al. 1986). cDNA cloning of the type I receptor has demonstrated sequence homology to the insulin receptor (Ullrich et al. 1986), although there are differences in the tyrosine residue structures of these receptors (Sahal et al. 1988). The type II receptor has a predicted molecular weight, from cDNA cloning, of 275 kD (Jaffe et al. 1983). This receptor binds IGF-I, the degree of stimulation being only up to 20% of that caused upon IGF-II binding (Rechler et al. 1980). The type II receptor does not exhibit tyrosine kinase activity although phosphorylation of receptor tyrosine residues occurs in vivo (Jaffe et al. 1983). The type II receptor has been demonstrated to have 99.4% homology with the mannose-6-phosphate receptor (Oshima et al. 1988), suggesting that this receptor is able to bind two distinct classes of ligands. For biological effects upon cells in culture, higher concentrations of IGF-II than IGF-I are required to produce the same response. This indicates that most biological responses are mediated via the type I receptor.

In both serum, and conditioned medium from cultured cells, IGFs are found in association with binding proteins (BPs). These BPs specifically bind IGF-I and IGF-II, but not insulin (Rechler and Nissley, 1990). Three separate, but structurally related BP groups have been reported. The first have a Mₐ of 28 kD and are termed IGF-BP1, the second class having a Mₐ of 53 kD (IGF-BP3). The cDNA for the IGF-BP1 (Brewer et al. 1988) and the IGF-BP3 (Wood et al. 1988) have been cloned. The other class of BP, IGF-BP2 although never having been isolated has a predicted Mₐ of 31.3 kD (Martin et al. 1990). Binding proteins have been detected in medium conditioned by various cell types including fibroblasts (Clemmons and Shaw, 1986) and endothelial cells (Bar et al. 1987). The IGF/BP complex is biologically inactive as it does not interact with receptors, probably
due to steric hindrance from the binding protein (Daughaday et al. 1980). Binding proteins have been shown to modulate the binding of IGF-I to cellular receptors, suggesting that they play an important role in regulating the cellular responses to IGF-I (De Vroede et al. 1986).

IGF-I mRNA has been reported as being detectable at the wound site *in vivo* (Steenfos and Jansson, 1992; Gartner et al. 1992), a number of sources including platelets, macrophages and fibroblasts (Bennett and Schultz, 1993b) having been described. IGF-I has been shown to stimulate: fibroblast migration (Khaw et al. 1994b); ECM contraction (Assouline et al. 1992; Khaw et al. 1994b); fibroblast proliferation (Khaw et al. 1994b) and ECM synthesis (Bird and Tyler, 1994; Khaw et al. 1994b). Roles for IGF-I in healing *in vivo* have also been shown, with this factor stimulating bone formation (Hock et al. 1988), regeneration of neural tissue following crush injury (Kanje et al. 1989; Sjoberg and Kanje, 1989) and in enhanced healing *in vivo* in combination with PDGF (Lynch et al. 1987).

1.5.7 Epidermal Growth Factor (EGF) and Transforming Growth Factor Alpha (TGFα)

A factor that stimulated both eyelid opening and eruption of incisors in new-born mice was discovered during the purification of nerve growth factor, and was later found to stimulate the growth of skin explants *in vitro* (Cohen, 1964). This factor was subsequently termed epidermal growth factor (EGF). EGF is a heat and acid stable molecule with a M₉ of 6 kD and an acidic isoelectric point (Taylor et al. 1972; Holladay et al. 1976). Transforming growth factor alpha (TGFα) was discovered, in conjunction with TGFβ, due to its ability to stimulate the anchorage-independent growth of fibroblasts (Roberts et al. 1987).
1980) *in vitro*. TGFα is a fifty amino acid single chain polypeptide with a $M_r$ of 5.6 kD (Derynck, 1988). TGFα is expressed by a variety of normal and malignant cell types. Secreted forms of TGFα exhibit a range of $M_r$ from 5-20 kD (Derynck, 1988), and the release of a 18-21 kD form which corresponds to the extracellular domain of the TGFα precursor has been reported (Ignotz et al. 1986b).

The size heterogeneity of these secreted molecules may reflect incomplete processing or differential glycosylation of the extracellular domain of the cell-associated pro-TGFα molecule (Salomon et al. 1990). Both EGF and TGFα are initially synthesised as precursors with a $M_r$ of 130 kD (Bell et al. 1986) and 25 kD (Derynck et al. 1984) respectively, which are then processed to yield 'mature' factors. These EGF and TGFα precursors are membrane-bound and exhibit biological activity (Mroczkowski et al. 1989; Wong et al. 1989) by binding to EGF receptors on adjacent cells, causing receptor autophosphorylation and signal transduction.

The EGF receptor was initially isolated and purified from preparations obtained from the A-431 cell line (Cohen et al. 1980; Cohen et al. 1982) which was derived from a human squamous cell carcinoma of the lung. The receptor is an intrinsic membrane glycoprotein having an extracellular domain with a total $M_r$ of 170kD. The intracellular domain of the receptor has been shown to exhibit tyrosine kinase activity (Buhrow et al. 1982). The binding of EGF to its receptor is rapidly followed by EGF-receptor-complex clustering and signal transduction, leading to a cellular response including an increase in mRNA for the EGF receptor itself (Haigler et al. 1979). The ligand-receptor complexes are eventually internalised and transported to lysosomes where both the ligand (Carpenter and Cohen, 1976) and the receptor (Stoscheck and Carpenter, 1984) are degraded.
In vitro, EGF has been shown to stimulate: the migration of fibroblasts (Adelmann-Grill et al. 1990; Grant et al. 1992; Khaw et al. 1994b), fibroblast-mediated ECM contraction (Assouline et al. 1992; Khaw et al. 1994b); fibroblast proliferation (Oka and Orth, 1983; Khaw et al. 1994b) and ECM production (Laato et al. 1987; Khaw et al. 1994b). The effects of EGF are perhaps the most studied in vivo of all growth factors. Exogenous EGF has been shown the accelerate cutaneous (Brown et al. 1991) and corneal tissue repair (Schultz, 1990) as well as increasing the tensile strength of wounds in pig stomach, ileum and duodenum (Kingsnorth et al. 1990). Additionally, EGF has been used in clinical trial and was shown to accelerate the rate of corneal healing by up to 30% compared to controls, following traumatic injury (Pastor and Calonge, 1992).

1.5.8 Transforming Growth Factor Beta (TGFβ)

The TGFβ molecules are a family of polypeptides which tend to be homodimers with a Mₙ of approximately 25kD and are stable to heat and acid treatment. The first TGFβ subtype, TGFβ₁, was purified from human platelets (Assoian et al. 1983). The second subtype was TGFβ₂ from tissues including: bovine brain (Seyedin et al. 1985), porcine platelets (Cheifetz et al. 1987) and human glioblastoma cells (Wrann et al. 1987). During the purification of TGFβ₂, a heterodimeric form of TGFβ, TGFβ₁₂, was also isolated (Cheifetz et al. 1987). A third major subtype of TGFβ, TGFβ₃, has been identified from cDNA library screening and subsequently cloned (Derynck et al. 1988). The degree of homology between the three TGFβ-subtypes ranges from 64% to 82% (Massague et al. 1992). Both TGFβ₁ and TGFβ₂ elicit similar effects in biological assays and are derived
from precursors consisting of 390 amino acids (Derynck et al. 1988) and 412 amino acids (de Martin et al. 1987) respectively. These precursors, once processed are secreted from cells in a predominantly latent form (LTGFβ) which is unable to bind receptors and is not recognised by TGFβ antibodies (Wakefield et al. 1988). The secretion of LTGFβ has been postulated to prevent intracellular ligand-receptor interaction (Wakefield et al. 1988). The structure of the LTGFβ₁ complex has been elucidated and has a molecular weight of approximately 220kD (Nilsen-Hamilton, 1990) consisting of a dimeric TGFβ molecule, a remnant of the precursor which upon removal of carbohydrate structures renders TGFβ active (Mlyazono and Heldin, 1989), and a TGFβ binding protein containing EGF-like sequences and possessing unknown function (Kanzaki et al. 1990).

Three membrane components which bind TGFβ with high affinity have been described, and are termed class I, II and III TGFβ receptors. Receptors for TGFβ are present on most cell types (Wakefield et al. 1987) although expression of the receptors varies, e.g., endothelial cells express only the class I and II receptors and not the class III receptors (Jennings et al. 1988; Segarini et al. 1989). Class I and II receptors have Mᵦ of 55 and 75 kD respectively (Cheifetz et al. 1986). The class III receptors are dimeric (Massague and Like, 1985) and consist of subunits with Mᵦ of 250 - 350 kD (Cheifetz et al. 1988). The class I and II receptors bind the different TGFβ isoforms preferentially, in the order of TGFβ₁>TGFβ₂>TGFβ₁₂ (Cheifetz et al. 1988). The class III receptors however bind the different TGFβ isoforms with equal affinity, a subset having been reported that have a higher affinity for TGFβ₂ (Segarini et al. 1987). The TGFβ receptors differ from the other growth factor receptors in that they exhibit serine/threonine kinase activity and not tyrosine kinase activity (Fanger et al. 1986). It has been suggested that a
heteromeric TGFβ receptor composed of both class I and II receptors is essential to signalling (Massague, 1992; Wrana et al. 1992; Inagaki et al. 1993). Subsequently it has been shown that the class II receptor, unlike the class I receptor, can bind ligands but the class I receptor is required for intracellular signalling to occur. This probably occurs via interactions between the intracellular regions of these receptors (Okadome et al. 1994).

TGFβ elicits a multitude of effects on cellular wound healing behaviour including the stimulation of leukocyte/monocyte and fibroblast migration (Ignotz et al. 1987; Grant et al. 1992; Khaw et al. 1994b); stimulation of fibroblast proliferation and ECM contraction (Ignotz et al. 1987; Khaw et al. 1994b); stimulation of ECM synthesis and inhibitors of remodelling enzymes as well as decreasing the production of these enzymes (Ignotz et al. 1987; Khaw et al. 1994b; Edwards et al. 1988); and the upregulation of integrin levels that may serve as to increase cell to matrix adhesion (Barzu et al. 1989). The major producers of TGFβ at the wound site are platelets, macrophages, keratinocytes, and fibroblasts (Bailey and Lovie, 1980). Additionally, the stimulation of fibroblasts with TGFβ has been reported to have an autoinductive effect on these cells (Kim et al. 1989). In vivo, TGFβ has been shown to be angiogenic, this effect appearing to be indirect as it has been reported that an increase in associated leukocyte recruitment occurs, probably resulting in the release of secondary angiogenic factors. Additionally, TGFβ increases wound healing, the strength of the healed tissue, cellularity and ECM formation in vivo with neutralisation of this molecule resulting in decreased scar tissue formation (Shah et al. 1992; Shah et al. 1994; Shah et al. 1995). In addition to the above effects, TGFβ also modulates the response of cells to other growth factors such as PDGF, FGF and EGF,
determining whether their effects are stimulatory or inhibitory (Roberts and Sporn, 1985; Sporn and Roberts, 1986). For example, TGFβ inhibits PDGF stimulated fibroblast proliferation, this effect appearing to result from a TGFβ mediated down-regulation of PDGF α receptors (Paulsson et al. 1993).

1.6 Growth Arrest

The regulation of cell homeostasis is via a combination of processes including proliferation, programmed cell death (apoptosis) and growth arrest (Williams, 1991; Marx, 1993). One of the major players in the process of growth arrest is the protein p53. This is a nuclear protein that functions as a transcriptional regulator (Vogelstein and Kinzler, 1992) that suppresses cell growth (Hartwell, 1992). The exposure of mammalian cells to agents such as those that damage DNA (e.g., chemotherapeutic agents) result in a series of cellular responses that evoke a reversible block in cell cycle progression at the G1 and G2 phases (Hartwell and Weinert, 1989). These blocks reflect the time for cells to repair the DNA damage before replication can occur. P53 has been implicated as playing a role in this pathway (Kastan et al. 1992) by stimulating the synthesis of cyclin-dependent kinase inhibitors such as p21/WAF and therefore halting cell cycle progression at G1 or G2. In addition to growth arrest occurring via p53-dependent pathways, p53-independent pathways have also been implicated in the process of growth arrest which are associated with several molecular mechanisms (see Figure 1.8 adapted from Liebermann et al. 1995).
Figure 1.8: Molecular mechanisms associated with growth arrest

p53 Dependent

- p53
- p21 (inhibition of cyclin dependent kinases)
- Retinoblastoma dephosphorylation (inhibition of G1-S phase transition)
- Growth arrest associated genes (inhibition of DNA synthesis and promotion of DNA repair)
- Bcl-2 (reduces cell survival and promotes apoptosis)

p53 Independent

- p21
- DNA damaging agents
- Growth arrest associated genes (induced by genotoxic stress, e.g., alkylating agents, radiation)
- Bcl-2

Growth Arrest Apoptosis

(Adapted from Liebermann et al, 1995)
1.6.1 Antimetabolites and their Mechanisms of Action

A number of chemotherapeutic or antiproliferative agents have been reported, and can be arbitrarily divided into groups according to their chemical nature and modes of action. These groups include antimetabolites, natural alkaloids, alkylating agents and differentiating agents (Figure 1.9; adapted from Tahery and Lee, 1989) and act at various stages of the cell cycle (Figure 1.10.). Members of the antimetabolite group of agents are highlighted in greater detail below.

Antimetabolites are agents that interfere with DNA/RNA synthesis (Blumenkranz et al. 1984), and are structural analogs to metabolically active molecules such as purines, pyrimidines and folic acid. 5-fluorouracil (5FU) is a pyrimidine analog, that is converted intracellularly to 5-fluoro-2-deoxyuridine (Figure 1.11; adapted from Lee et al. 1991) and inhibits the action of thymidylate synthetase which is required for DNA synthesis. Additionally 5FU is also incorporated into RNA resulting in the production of abnormal ribosomes, altered mRNA translation and abnormal protein synthesis (Rich et al. 1960; Madoc-Jones and Bruce, 1968; Gressel et al. 1984). In relation to the process of wound healing, 5FU has been shown to inhibit fibroblast proliferation (i.e., cause growth arrest) in vitro without causing cell death, the effects being long-term (in excess of 36 days) and apparent even with single exposures as short as five minutes (Khaw et al. 1992b; Khaw et al. 1992c). In vivo, exposure to this agent has also been shown to reduce scarring in animal models and humans following glaucoma filtration surgery, the effects being long term, focal and titratable (Khaw et al. 1992a; Doyle et al. 1993; Khaw et al. 1993a; Khaw et al. 1993b). Mitomycin-C (MMC) is a natural alkaloid that has multiple actions including the alkylation and crosslinking of DNA (Iyer and Szybalski, 1963), free radical formation
(Moore, 1977) and ultimately an inhibition of RNA and subsequent protein synthesis (Lerman and Benyumovitch, 1965). Like 5FU, exposure to MMC has been shown to inhibit fibroblast proliferation in vitro in addition to reducing scarring in vivo with single exposures as short as five minutes (Khaw et al. 1992b; Khaw et al. 1993a; Khaw et al. 1993b). The success of glaucoma filtration surgery, to primarily reduce the increased intraocular pressure associated with glaucoma, is dependent upon the degree of post-operative scarring. The above experimental findings have been found to have significant clinical importance in the reduction of post-operative scarring in humans, following glaucoma filtration surgery. However, certain patients still fail surgery due to scarring even though they received antimetabolites (The Fluorouracil Filtering Surgery Study Group, 1996). The reasons for this are still unclear. Although the effects of single five minute exposures to these agents upon fibroblast proliferation in vitro and wound healing in vivo have been extensively studied, and shown to cause long term fibroblast growth arrest, their effects upon other fibroblast functions crucial to the scarring process are currently unclear. The possible ability of these growth arrested cells to still perform scarring functions is obviously important to the modulation of tissue repair in vivo, and may occur as scarring is sometimes seen following exposures to these agents.

Additionally, human diploid epidermal keratinocytes are serially cultivated on layers of growth arrested 3T3 fibroblast feeder layers, a technique routinely used in many laboratories. The original methods described by Rheinwald and Green for the productionn of these feeder layers included inducing growth arrest by radiation (Rheinwald and Green, 1975) or exposure to MMC (Rheinwald, 1980). Although these methods are still in use, over twenty years later, the exact mechanisms of how they support keratinocyte growth are unclear. A report by Blacker et al (1987), suggested that 3T3 fibroblasts were capable
of producing eicosanoids (prostaglandin E₂ and 6-keto-prostaglandin F₁α) even though growth arrested. This group suggested that these molecules may contribute to the support of keratinocyte growth. It therefore appears that growth arrested feeder layers do not just serve as an inert structural matrix for epidermal cell growth, but may produce molecules that influence their behaviour.
### Figure 1.9: Chemotherapeutic/antiproliferative agents and their modes of action

<table>
<thead>
<tr>
<th>Group</th>
<th>Drug</th>
<th>Mode of action</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antimetabolites</td>
<td>Fluorouracil</td>
<td>Converted to the active form fluorodeoxyuridine monophosphate (FdUMP), which inhibits thymidylate synthetase</td>
</tr>
<tr>
<td>Natural Alkaloids</td>
<td>Mitomycin-C</td>
<td>DNA fragmentation, inhibition of dependent RNA synthesis, membrane disruption, free radical formation and metal chelation</td>
</tr>
<tr>
<td>Alkylating Drugs</td>
<td>Chlorambucil</td>
<td>Induction of alkyl groups into proteins and nucleic acids by covalent bonding</td>
</tr>
<tr>
<td></td>
<td>Carmustine</td>
<td></td>
</tr>
<tr>
<td>Differentiating</td>
<td>Sodium butyrate</td>
<td>Cause conformational change in DNA through inhibition of histone deacetylation</td>
</tr>
<tr>
<td>Agents</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure highlights specific agents within the major groups of chemotherapeutic / antiproliferative agents and their modes of action.

(Adapted from Tahery and Lee, 1989)
Figure 1.10: The cell cycle and chemotherapeutic agents

<table>
<thead>
<tr>
<th>Cell cycle</th>
<th>Affecting Agent</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M phase</strong> (mitosis)</td>
<td>Natural alkaloids</td>
</tr>
<tr>
<td></td>
<td>e.g., mitomycin-C</td>
</tr>
<tr>
<td><strong>G0 phase</strong> (quiescent)</td>
<td>Alkylating agents</td>
</tr>
<tr>
<td></td>
<td>e.g., carmustine</td>
</tr>
<tr>
<td><strong>G1 phase</strong> (enzymes needed for DNA synthesis)</td>
<td>Alkylating agents</td>
</tr>
<tr>
<td></td>
<td>e.g., chlorambucil</td>
</tr>
<tr>
<td></td>
<td>Natural alkaloids</td>
</tr>
<tr>
<td></td>
<td>e.g., mitomycin-C</td>
</tr>
<tr>
<td><strong>S phase</strong> (DNA synthesis)</td>
<td>Antimetabolites</td>
</tr>
<tr>
<td></td>
<td>e.g., 5-fluorouracil</td>
</tr>
<tr>
<td><strong>G2 phase</strong> (RNA synthesis)</td>
<td>Natural alkaloids</td>
</tr>
<tr>
<td></td>
<td>e.g., mitomycin-C</td>
</tr>
</tbody>
</table>
Figure 1.11: Biochemical mechanism of action of fluoropyrimidine

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Substrate products</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 uridine phosphorylase</td>
<td>FU, FUMP, FdUR</td>
</tr>
<tr>
<td>2 uridine kinase</td>
<td>FUMP, 5-fluoro-5 monophosphate</td>
</tr>
<tr>
<td>3 phosphoribosyl transferase</td>
<td>FdUMP, 5-fluoro-2 deoxyuridine</td>
</tr>
<tr>
<td>4 thymidine phosphorylase</td>
<td>FdUMP, 5-fluoro-2 deoxy-5 monophosphate</td>
</tr>
<tr>
<td>5 thymidine kinase</td>
<td>FUDP, 5-fluorouridine-5 diphosphate</td>
</tr>
<tr>
<td>6 thymidylate synthetase</td>
<td>FUTP, 5-fluorouridine-5 triphosphate</td>
</tr>
<tr>
<td>7 pyrimidine monophosphate kinase</td>
<td>FdUDP, 5-fluorodeoxyuridine-5 diphosphate</td>
</tr>
<tr>
<td>8 pyrimidine diphosphate kinase</td>
<td>FdUTP, 5-fluorodeoxyuridine-5 triphosphate</td>
</tr>
<tr>
<td>9 ribonucleotide reductase</td>
<td>dUMP, Deoxyuridine-5 monophosphate</td>
</tr>
<tr>
<td>10 RNA polymerase</td>
<td>dTMP, Deoxythymidine-5 monophosphate</td>
</tr>
<tr>
<td></td>
<td>dTTP, Deoxythymidine-5 triphosphate</td>
</tr>
</tbody>
</table>

The fluorinated pyrimidines produce their inhibitory and cytotoxic effects by two separate biochemical mechanisms involving RNA and DNA.

(Adapted from Lee et al, 1991)
1.7 Justification and Aims

In the context of previous research, the fibroblast has been identified as the key player in the wound healing response following tissue injury. These cells carry out a number of crucial molecular and cellular functions that contribute to the overall healing response, which are controlled by a number of extracellular stimuli. Several studies have demonstrated that modulation of one of these fibroblast functions, proliferation, via single, short exposures to antimetabolites results in long term growth arrest and reduction of scarring in vivo, although not in every case. However, what is currently unclear is the effects of these single exposures to antimetabolites on the ability of fibroblasts to carry out molecular and cellular functions related to the healing process (other than proliferation). Additionally, growth arrested fibroblast feeder layers are used to support the growth of keratinocyte cultures, a technique that has been used for over twenty years. The mechanisms underlying the support of keratinocyte growth by growth arrested fibroblasts is unclear. Thus, the aim of this thesis was to investigate the long term effects of antimetabolite induced growth arrest on fibroblast wound healing behaviour including:

a) Growth factor production and growth factor receptor expression
b) Extracellular matrix production
c) Cellular migration
d) Extracellular matrix contraction.

It was hoped that the findings in this study would contribute to an increased understanding of the basic science of wound healing, and additionally reveal findings that may be clinically important in the modulation of the healing process.
2. METHODS AND MATERIALS

2.1 Cell Culture

2.1.1 Establishment of Tenon's Capsule Fibroblast Cultures

Pieces of Tenon's capsule were dissected off human donor eyes from Moorfields Eye Hospital Eye Bank. The tenets of the Declaration of Helsinki (1989) were followed, informed consent was obtained, and institutional human experimentation committee approval was granted. This tissue explant was then placed into a 25cm² tissue culture flask (Marathon), and anchored to the bottom of the flask with a 0.5cm² sterile round glass coverslip (BDH/Merck). Explants were then overlaid with 5ml of Dulbecco's modified Eagles medium (DMEM) containing penicillin (100U/ml), streptomycin (100μg/ml), gentamicin (50μg/ml), fungizone (0.25μg/ml), 2mM L-glutamine and supplemented with 10 % (vol/vol) newborn calf serum (NCS) (all Gibco). Fibroblasts had migrated out of the explant and started to proliferate by 7 days post-seeding. Once the cells were nearly confluent (usually within 3-4 weeks) they were passaged into new flasks and maintained in culture, stored in liquid nitrogen (see Section 2.1.4) or used for experimentation.

2.1.2 Routine Maintainence of Fibroblast Cultures

Flasks containing primary cultures were maintained by changing the growth medium every 3 to 4 days until the cells were nearing confluence. The monolayers were then passaged (see Section 2.1.3) into new tissue culture flasks. The routine of changing medium and passaging was continued until passage 6, with frequent storing of aliquots of cells in liquid nitrogen for future use (see Section 2.1.4).
Cultures were also screened for the presence of *Mycoplasma*, as these organisms have been found to interfere with a variety of cellular processes thus invalidating any experimental data obtained using infected cultures. These organisms cannot be detected visually in cultures and therefore regular screening for contamination is required (McGarrity, 1976). The mycoplasma detection system used routinely for screening was the Gen-Probe mycoplasma T.C. system (Lab Impex). Cultures were prepared for screening by growth in antibiotic-free medium for a total of two medium changes, samples of medium being exposed to cells for three days prior to being tested using the procedure supplied with the kit. The assay system employed the use of a ^3^H-labelled cDNA probe to mycoplasma ribonucleic acid (RNA; reactive with twenty species of *Mycoplasma*). The labelled cDNA probe forms stable DNA:RNA hybrids in infected samples, the quantity of which are determined by scintillation counting. A result of >0.4% hybridisation indicated an infected culture.

2.1.3 Passaging of Cultures

Cell monolayers were grown until just preconfluent. Growth medium was aspirated and the cells were removed from their substratum by incubation for 3 minutes at 37°C with a 0.15% (vol/vol) phosphate buffered trypsin/0.02% (wt/vol) ethylenediamine tetra-acetic acid (EDTA) solution (Gibco). The enzymic activity of this solution was then neutralised with an equal volume of growth medium containing NCS. This solution was then centrifuged at 200g for 8 minutes. The supernatant was discarded and the cell pellet resuspended in growth medium. The cells were then seeded into new tissue culture flasks at a split ratio of 1:3, i.e, one flask of cells was split into three new flasks.
2.1.4 Storage and Recovery of Cells in Liquid Nitrogen

Cultures of human Tenon's capsule fibroblasts were passaged and the resultant cell pellet resuspended in 0.5ml of growth medium. An equal volume of 10% (vol/vol) dimethyl sulphoxide (DMSO; Sigma) / growth medium was added dropwise with continual mixing. These 1ml aliquots of cell suspension were then transferred to cryovials (Nunc, Gibco) and stored overnight in the vapour phase of liquid nitrogen stores. The vials were then stored under liquid nitrogen until required.

Upon removal from liquid nitrogen, the contents of cryovials were rapidly thawed under hot, running water (60°C). The 1ml aliquot of cell suspension was then transferred to a sterile centrifuge tube and 5ml of growth medium was added dropwise, at a rate of 1ml per minute, with continual mixing. The cell suspension was then centrifuged at 200g for 8 minutes, the supernatant discarded and the cell pellet resuspended in growth medium and seeded into tissue culture flasks.

2.2. Exposure to Antimetabolites

The growth medium overlying cultures was removed prior to the addition of 5FU (0.25-25mg/ml; David Bull Laboratories), MMC (0.001-0.1 mg/ml; Kyowa) or phosphate buffered saline (PBS, pH 7.4; Unipath Ltd). The cultures were then incubated for 5 minutes at 37°C, the solution aspirated and the cultures washed with PBS a total of five times prior to the addition of fresh growth medium.

2.3 Isolation and Quantitation of Total Cellular RNA

Total RNA was isolated from cell cultures exposed in triplicate to antimetabolites (5FU and MMC) and PBS (controls) days 3, 7, 14, 24, 36 and 48 post treatment using the
RNEasy total RNA purification kit (Qiagen). This kit is based upon the guanidium thiocyanate method originally described by (Chomczynski and Sacchi, 1987), and was chosen in preference to the original method as the processing of large sample numbers proved to be easier technically, in addition to the purity and quantity of the RNA harvested being comparable to the original method. Briefly, medium overlying the cell monolayers was removed and the cells harvested by trypsinisation and centrifugation as described in Section 2.1.3. The resultant cell pellets were then directly lysed with 350μl of lysis buffer RLT (supplied with the kit) containing 1% (vol/vol) β-mercaptoethanol (Sigma) and several passages through a 25 guage needle attached to a 1ml syringe, to shear the nucleic acids within the sample. An equal volume of 70% (vol/vol) ethanol in RNAse free water (supplied with the kit) was added to the solution, mixed gently and applied to an RNEasy spin column followed by centrifugation at 8000g for 15 seconds. Sample RNA bound to the column was then washed with 700μl of wash buffer RW1 (supplied with the kit) and centrifuged at 8000g for 15 seconds. The spin column was then transferred to a new 2ml collection tube and washed with 500μl of wash buffer RPE via centrifugation at 8000g for 15 seconds. The spin column was then washed again with 500μl of wash buffer RPE (supplied with the kit) via centrifugation at 10000g for 2 minutes and transferred to a new collection tube. Column bound RNA was then eluted with 30μl of RNAse free water by centrifugation at 8000g for 1 minute. The concentration and purity of the eluted RNA was determined by measuring the absorbance of the solution at 260nm (A260; nucleic acids) and 280nm (A280; proteins) on a GeneQuant spectrophotometer (Pharmacia). Only RNA with a purity ratio of (A260:A280) 1.7-2.1 was used for experimentation. The cell numbers in each sample were calculated as described in Section 2.6.2, from sister flasks.
seeded with the same initial cell density, from the same cell suspension and growth arrested at the same time. The quantity of RNA produced in each sample was expressed as the mean quantity of RNA/ 10^5 cells (µg/10^5 cells, +/- S.E.) of triplicate samples. The differences in RNA production were statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.

2.4 Quantitation of Protein Production

Cells were seeded into 25cm² flasks (10000 cells/cm²) and allowed to settle overnight. The cells were then exposed to antimetabolites 5FU (0.25-25mg/ml) and MMC (0.001-0.1mg/ml) or PBS as described in Section 2.2. The monolayers were then washed (3 x 10ml) in PBS, followed by two changes in serum-free medium (4 hours each change, to ensure residual serum depletion) and finally incubated for 24 hours at 37°C in phenol red/serum-free DMEM (Sigma) days 3, 7, 14, 24, 36 and 48 post-exposure to antimetabolites. As the protein assay used in this series of experiments was a colourimetric method, phenol red-free medium was used upon the cultures to eliminate any interference during sample optical density determination. Samples of conditioned medium from these cultures were filter sterilised through a 0.2µm filter (Sartorius) to remove debris and insoluble material and then assayed for total protein using the Bio-Rad Protein Microassay (Bio-Rad). This protein assay was chosen as it not only used small volumes of samples (5µl), but that it also allowed rapid sample screening (within 30 minutes). A standard curve was prepared by diluting bovine serum albumin (BSA; Sigma) in PBS, at concentrations ranging from 36.25µg - 4mg/ml. Aliquots (5µl) of standards and samples
were pipetted in triplicate into a clean, dry microtitre plate. To this, 25μl/well of reagent A (alkaline copper tartrate solution) was added followed by the addition of 200μl of reagent B (dilute Folin reagent). The plate was then gently agitated to mix the reagents, followed by incubation for 15 minutes at room temperature. The optical densities of each well were then read at 650nm using a microplate reader (Titertek Plus MS2 reader; ICN Flow) and the values for each standard expressed as the mean (+/- S.E.) of triplicate wells. A standard curve of protein concentration versus optical density at 650nm was plotted and an equation for this standard curve determined using a polynomial trendline fit, generated using computer software (Microsoft Excel). The levels of protein in triplicate conditioned medium samples were calculated from this standard curve equation and expressed as the mean (+/- S.E.). The total cell number for each sample was also determined as described in Section 2.6.2, allowing calculation of mean quantity of protein (μg) / 10^3 cells (+/- S.E.). The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.
2.5 Quantitative Competitive Reverse Transcriptase Polymerase Chain Reaction (QCRT-PCR) Technique

2.5.1 Synthetic Template Construction for mRNA Analysis of Growth Factors, Growth Factor Receptors, Extracellular Matrix Molecules, Matrix Metalloproteinases (MMPs) and the Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) by QCRT-PCR

The production of growth factors, ECM molecules, proteins that are involved in turnover of the ECM (MMPs and TIMPs) and the expression of growth factor receptors all play a crucial role in the wound healing process (see Section 1). All of these proteins are produced by the induction of specific genes, followed by the transcription of these genes to produce specific messenger RNAs (mRNAs), which are then translated into the corresponding proteins. The detection of specific mRNAs within cells has been achieved using a variety of probe hybridization techniques e.g., Northern blotting and in situ hybridisation. However, the polymerase chain reaction (PCR) allows a more sensitive and specific analysis of nucleotide sequences, although only from DNA or cDNA samples.

Amplification of mRNA by the polymerase chain reaction (PCR) following reverse transcription (RT) has become a widely accepted technique for the detection of mRNA in both cells and tissues (Chelly et al. 1988; Delidow et al. 1989). Quantitation of mRNA by RT-PCR has been attempted using endogenous internal standards or "housekeeping genes" such as β-actin. Unfortunately, inter- and intra-assay variation, either due to efficiency of amplification of individual primer pairs or actual variations in the levels of the standard within different samples, are often too great to be reliable (Hoof et al. 1991; Murphy et al. 1990). The development of quantitative competitive based RT-PCR (QCRT-PCR) strategies have allowed a more accurate and reproducible quantitation of specific mRNAs.
Therefore, a QCRT-PCR technique was chosen for use in this study (Tarnuzzer et al. 1996; see Figure 2.3 for an overview), which involved the use of synthetic RNA templates for growth factors, growth factor receptors, ECM degrading enzymes and their inhibitors and ECM molecules consisting of sequences complimentary to those for the 5' and 3' primers from published sequences (see Table 2.1). The design of the templates was such that the amplified template had a smaller base pair (bp) size than that of the amplified message from cellular/tissue mRNA. This allowed separation and direct comparison of the template and sample products (see below and Table 2.2). Serial dilutions of this template were made so that a range of initial copies (from $6.8 \times 10^3$ - $6.8 \times 10^8$ copies) for each particular message could be measured. These template dilutions were combined into reaction tubes with constant amounts of sample RNA. This mixture then underwent a reverse transcription reaction which made cDNA copies of the RNA (both sample and template) in each tube. Specific primers for each message of interest were then added to sets of tubes containing aliquots of the RT reaction, followed by amplification by PCR. In this reaction both the synthetic template and the sample RNA competed for primer binding, thus setting up a competitive reaction. The samples were then separated by agarose gel electrophoresis, and the intensities of the amplified sample (number of initial copies unknown) and amplified synthetic template (initial copy numbers from $6.8 \times 10^2$ - $6.8 \times 10^8$ copies) quantitated by image analysis. The log value of the ratio of amplified template to amplified sample was then plotted versus the log value of the initial copy number of the template. When the ratio of the intensity of the amplified template to sample was equal to one, then the initial copy numbers were equal. During the quantitation of growth factor, growth factor receptor, ECM molecule, MMP and TIMP expression, the detection of β-actin using specific primers was also carried out. This was because the
quantitation of RNA by spectrophotometry, described in Section 2.3, does not indicate if the RNA isolated is intact or degraded. Therefore as β-actin is always expressed at some level in all cells, detection of this mRNA in samples was employed as an internal positive control to ensure that the absence of any particular mRNA in samples was due to actual lack of expression and not to total RNA degradation.

Using this QCRT-PCR system it was possible to combine several of the synthetic templates with the initial RNA samples prior to the RT reaction, allowing the analysis of several mRNA species from the same cellular/tissue RNA sample. In addition, this system was extremely powerful as it allowed rapid, quantitative, multiple message analysis from small quantities of sample RNA (0.1 - 1μg). All synthetic templates used in this study were kind gifts of Dr. Roy W. Tarnuzzer (University of Florida, USA), their construction and preparation being outlined in the following sections.

2.5.2 Synthetic Template Plasmid Construction

Several synthetic templates for growth factors, growth factor receptors, ECM molecules, MMPs and TIMPs have been used in this study and are illustrated in Figure 2.1. Although the primer sequences for each message are different, the general design and method of template production are the same for all templates. The sources of primer sequences are given in Table 2.1 and the actual sequences of the primers used are given in Table 2.2.

Construction of the template plasmids (see Figure 2.2) was using a modification of the method described by Dillon and Rosen (1990). Briefly, eight overlapping 79 base oligonucleotides containing the complimentary sequences to the 3', 5' and the internal PCR primers were synthesised. Five hundred nanograms of each of the template oligonucleotides
were mixed in a standard PCR (100μl) containing 200μM of each dinucleotide triphosphates (dNTP; Promega), 2.5U of Taq DNA polymerase (Perkin-Elmer), 50mM KCl, 10mM Tris-HCl (pH 8.0; Perkin-Elmer), 1.5mM MgCl₂ (Perkin-Elmer) with no additional template or primers. The PCR reaction was carried out for 7 cycles (94°C for 7 minutes, 55°C for 2 minutes and 72°C for 3 minutes) using a thermocycler (Omnigene, Hybaid). One microlitre of this reaction was added to a second standard PCR containing 1μg of the flanking 21 base oligonucleotides and amplified for 25 cycles (see above). Ten microlitres of this reaction was separated on a 2% (wt/vol) low melting agarose gel dissolved in tris-acetate (0.04M)/EDTA (0.001M) buffer, pH 8, and the amplified band was excised. The gel slice containing the template DNA was heated to 65°C for 10 minutes, cooled to 37°C and combined with 1μg of pCRII plasmid DNA, ligation buffer, 15U T4 DNA ligase (Promega) and incubated at room temperature for 18 hours. The ligated material was isolated from the agarose by phenol:chloroform extraction (Chomczynski and Sacchi, 1987) followed by ethanol precipitation and resuspension in 10μl of tris-acetate (0.04M)/EDTA (0.001M) buffer, pH 8. Twenty microlitres of INVα competent cells were transformed with 2μl of the reconstituted ligation mix in a standard transfection protocol (Sambrook et al. 1989) DNA was isolated from a positive colony and a poly (A)₈ tail oligonucleotide cassette was cloned into a BamHI-HindIII site at the 3' end of the template by standard protocols (Sambrook et al. 1989). DNA was isolated from a positive clone and purified by caesium chloride gradient centrifugation.
Figure 2.1: Synthetic templates constructed for QCRT-PCR analysis of growth factor, growth factor receptor, extracellular matrix molecule, matrix metalloproteinase and tissue inhibitors of matrix metalloproteinases mRNA production

**Growth factors/ Growth factor receptors**

<table>
<thead>
<tr>
<th>Template 1 (Designated pEGF/TGF)</th>
<th>Template 2 (Designated pPDGF/FGF)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epidermal growth factor</td>
<td>Platelet derived growth factor A</td>
</tr>
<tr>
<td>Transforming growth factor β3</td>
<td>Platelet derived growth factor B</td>
</tr>
<tr>
<td>Epidermal growth factor receptor</td>
<td>Acidic fibroblast growth factor</td>
</tr>
<tr>
<td>Transforming growth factor β type II receptor</td>
<td>Platelet derived growth factor receptor α</td>
</tr>
<tr>
<td>Transforming growth factor α</td>
<td>Platelet derived growth factor receptor β</td>
</tr>
<tr>
<td>Transforming growth factor β1</td>
<td>Basic fibroblast growth factor receptor</td>
</tr>
<tr>
<td>Transforming growth factor β2</td>
<td>Basic fibroblast growth factor receptor</td>
</tr>
<tr>
<td>β-actin</td>
<td>Acidic fibroblast growth factor receptor</td>
</tr>
</tbody>
</table>

**Matrix metalloproteinases / Tissue inhibitors of matrix metalloproteinases**

<table>
<thead>
<tr>
<th>Template 3 (Designated pMMP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP 9 (92kD gelatinase)</td>
</tr>
<tr>
<td>MMP 2 (72kD gelatinase)</td>
</tr>
<tr>
<td>MMP 3 (stromelysin)</td>
</tr>
<tr>
<td>Neutrophil elastase</td>
</tr>
<tr>
<td>MMP 1 (collagenase)</td>
</tr>
<tr>
<td>TIMP 1 (tissue inhibitor of matrix metalloproteinases 1)</td>
</tr>
<tr>
<td>TIMP 2 (tissue inhibitor of matrix metalloproteinases 2)</td>
</tr>
<tr>
<td>MMP 7 (putative metalloproteinase-1; PUMP-1)</td>
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</table>

**Extracellular matrix molecules**

<table>
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<tr>
<th>Template 4 (Designated pECM)</th>
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<tbody>
<tr>
<td>Collagen type I</td>
</tr>
<tr>
<td>Collagen type III</td>
</tr>
<tr>
<td>Collagen type IV</td>
</tr>
<tr>
<td>Elastin</td>
</tr>
<tr>
<td>Fibronectin</td>
</tr>
<tr>
<td>Lysyl oxidase</td>
</tr>
<tr>
<td>Laminin B1</td>
</tr>
<tr>
<td>Laminin B2</td>
</tr>
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Table 2.1: Sources of primers used for synthetic RNA template construction and QCRT-PCR

<table>
<thead>
<tr>
<th>Synthetic Templates</th>
<th>Author</th>
<th>Journal</th>
<th>Year/Vol/Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transforming Growth Factor β3</td>
<td>ten Dijke et al</td>
<td>PNAS</td>
<td>1988: 85; 4715</td>
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<tr>
<td>Epidermal Growth Factor Receptor</td>
<td>Xu et al</td>
<td>Nature</td>
<td>1982: 309; 806</td>
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<tr>
<td>Transforming Growth Factor β type II Receptor</td>
<td>Yin et al</td>
<td>Cell</td>
<td>1992: 68; 775</td>
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<tr>
<td>Transforming Growth Factor α</td>
<td>Derynck et al</td>
<td>Cancer Cells</td>
<td>1989: 301</td>
</tr>
<tr>
<td>Transforming Growth Factor β1</td>
<td>Derynck et al</td>
<td>Nature</td>
<td>1985: 316; 701</td>
</tr>
<tr>
<td>Transforming Growth Factor β2</td>
<td>de Matin et al</td>
<td>EMBO J</td>
<td>1989: 6; 3673</td>
</tr>
<tr>
<td>β-actin</td>
<td>Ng et al</td>
<td>Mol Cell Biol</td>
<td>1985: 5 (10); 2720</td>
</tr>
<tr>
<td>Platelet Derived Growth Factor B</td>
<td>Collins et al</td>
<td>Nature</td>
<td>1985: 316; 748</td>
</tr>
<tr>
<td>Acidic Fibroblast Growth Factor Receptor</td>
<td>Chia et al</td>
<td>Oncogene</td>
<td>1990: 5; 755</td>
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<tr>
<td>Platelet Derived Growth Factor Receptor α</td>
<td>Matsui et al</td>
<td>Science</td>
<td>1989: 243; 800</td>
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<tr>
<td>Platelet Derived Growth Factor Receptor β</td>
<td>Claesson-Welch</td>
<td>Mol Cell Biol</td>
<td>1988: 8; 3476</td>
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<tr>
<td>Basic Fibroblast Growth Factor Receptor</td>
<td>Dionne et al</td>
<td>EMBO J</td>
<td>1990: 9; 2685</td>
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<td>Acidic Fibroblast Growth Factor Receptor</td>
<td>Avivi et al</td>
<td>Oncogene</td>
<td>1991: 6; 1089</td>
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<td>MMP 9 (92 kD gelatinase)</td>
<td>Sims et al</td>
<td>Biochemistry</td>
<td>1992: 31; 7120</td>
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<td>MMP 2 (72kD gelatinase)</td>
<td>Collier et al</td>
<td>J Biol Chem</td>
<td>1988: 263; 6579</td>
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<td>MMP 3 (stromelysin)</td>
<td>Saus et al</td>
<td>J Biol Chem</td>
<td>1988: 263; 6742</td>
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<td>MMP 1 (collagenase)</td>
<td>Goldberg et al</td>
<td>J Biol Chem</td>
<td>1986: 261; 6600</td>
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<td>TIMP 1 (Tissue Inhibitor of Matrix Metalloproteinases 1)</td>
<td>Docherty et al</td>
<td>Nature</td>
<td>1985: 318; 66</td>
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<td>TIMP 2 (Tissue Inhibitor of Matrix Metalloproteinases 2)</td>
<td>Stetler-Stevenson</td>
<td>J Biol Chem</td>
<td>1990: 265; 13933</td>
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<td>Collagen type I</td>
<td>de Wet et al</td>
<td>J Biol Chem</td>
<td>1987: 262; 16032</td>
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<td>Collagen type III</td>
<td>Emanuel et al</td>
<td>PNAS</td>
<td>1985: 82; 3385</td>
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<td>Collagen Type IV</td>
<td>Soininen et al</td>
<td>FEBS Lett</td>
<td>1987: 225; 188</td>
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<td>Elastin</td>
<td>Fazio et al</td>
<td>J Invest Dermatol</td>
<td>1988: 91; 458</td>
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<tr>
<td>Fibronectin</td>
<td>Bernard et al</td>
<td>Biochemistry</td>
<td>1985: 24; 2698</td>
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<tr>
<td>Lysyl oxidase</td>
<td>Hamalainen et al</td>
<td>Genomics</td>
<td>1991: 11; 508</td>
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(Information for table kindly provided by Dr. Roy Tamuzer, University of Florida).
<table>
<thead>
<tr>
<th>mRNA</th>
<th>5' (antisense) Primer</th>
<th>3' (sense) Primer</th>
<th>Size</th>
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</thead>
<tbody>
<tr>
<td>EGF</td>
<td>AGCGTGCCGCGATCCCTACAC</td>
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<td>TGF β3</td>
<td>GCCGCCCTTTTCTCTCTGCCTC</td>
<td>ATTACCTACAGTTTTCCCGG</td>
<td>541</td>
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<tr>
<td>EGF receptor</td>
<td>AAACCATGTCTGTGGGCTCAAG</td>
<td>GTGACCCTTTGGAGGTGATG</td>
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<tr>
<td>TGF β type II receptor</td>
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<td>TGTGGCTCTTGAGTTCTGAG</td>
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<td>TGF α</td>
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<td>TGF β1</td>
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<td>TGF β2</td>
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<td>CACCTCTGCTCAGTGAGC</td>
<td>603</td>
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<tr>
<td>β-actin</td>
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<td>GTGGGGCTGCTGCTGAGCA</td>
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<tr>
<td>PGDF A</td>
<td>AATCCGACGATCCGCTTCGCT</td>
<td>TGGCTGGCTGCTGCTGCTC</td>
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<tr>
<td>PDGF B</td>
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<td>GCTGGCAGCTTCTCTGCTC</td>
<td>555</td>
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<tr>
<td>aFGF</td>
<td>GCGACGCCAATGTCAGTGAAG</td>
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<td>590</td>
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<tr>
<td>PDGF receptor α</td>
<td>CAGCATTGTGCTAGCCTTCCCT</td>
<td>AGAGTCTCCGATGGAACAC</td>
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<td>PDGF receptor β</td>
<td>AGTCAAGCGGCTCCACACGCG</td>
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<tr>
<td>bFGF receptor</td>
<td>TGTTACTGGCTCCACTAGCACA</td>
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<td>bFGF</td>
<td>GAATTCGAGTATAGATGTCCT</td>
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<td>545</td>
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<td>aFGF receptor</td>
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<tr>
<td>MMP 9</td>
<td>AAACCCGCTCTCCCTCTGCTC</td>
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<td>MMP 2</td>
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<tr>
<td>MMP 3</td>
<td>GTCTGGAGAGGACAGTGTTGCT</td>
<td>TCAGAATGCTTCTTGGGCTGGC</td>
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<td>TTGTCTCTCAGTAGGAAAC</td>
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<td>Neutrophil elastase</td>
<td>TGCCACCGGCGGAAAGGACAT</td>
<td>GCACGCGTGTCGGCGGCGAG</td>
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<tr>
<td>TIMP 1</td>
<td>GACACTGTGCGAGCCTGACT</td>
<td>CAGACACCTTTATACAGC</td>
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<tr>
<td>TIMP 2</td>
<td>GTTGAGCCGCTGTTATGTCG</td>
<td>TCCTGGGAGACAGCTATGAG</td>
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<td>MMP 7 (PUMP-1)</td>
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<td>TACTCTACTATTTCCCAATAG</td>
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<td>Collagen type III</td>
<td>CCATCTGCGCAGGTTCTCC</td>
<td>GGCTCTGGTGGAGGAGGAG</td>
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<tr>
<td>Fibronectin</td>
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<td>CCGGGCTGCTGCTGAGAAGC</td>
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<tr>
<td>Collagen type IV</td>
<td>TGCTGTCCAGGAAGGCCAGG</td>
<td>GGAGAAGCAGGCTTCTGGA</td>
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<tr>
<td>Elastin</td>
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<tr>
<td>Lysyl oxidase</td>
<td>CTCGGATGATAGGTGATGCTG</td>
<td>GGGAGAAGCAGGCTGAG</td>
<td>528</td>
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<tr>
<td>Laminin B1</td>
<td>CCGTGCAACATTCCTTACTC</td>
<td>CTGCGGAGGAGGAGGAG</td>
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<tr>
<td>Laminin B2</td>
<td>CGCCGGCTCATGGAAGAAG</td>
<td>GGGCGGCTCCTGAGCAGACT</td>
<td>677</td>
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</tbody>
</table>

| PEFG/TGF | ---- | ---- | 337 |
| pPDGF/FGF | ---- | ---- | 362 |
| pMMP | ---- | ---- | 333 |
| pECM | ---- | ---- | 347 |

(Information for table kindly provided by Dr. Roy Tamuzzer, University of Florida).
2.5.3 Preparation of Competitive RNA Templates

Each competitive template RNA was generated from the template plasmid by transcription with either T7 polymerase after linearisation with HindIII (Promega), or SP6 RNA polymerase (Promega) (see Figure 2.2) after Xba I digestion (Promega). This reaction mix was digested with Proteinase K (Promega) at 37°C for 1 hour, extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (1:1:0.2). A typical T7 transcription reaction contained 1μg of linearised plasmid DNA template, 7.5mM of each dNTP, 1U of human placental ribonuclease inhibitor, and 10U of T7 polymerase in 40mM Tris-HCl (pH 7.9), 10mM NaCl, 30mM DTT, 6mM MgCl₂, 2mM spermidine and 50mM EDTA. The reaction was carried out at 37°C for 1 hour and the poly (A)ₜ template was purified by oligo (dT) chromatography (Promega). The total copy number of each template generated was determined as follows. Quantitation of the total mass of RNA produced (μg) was achieved by spectrophotometry (see Section 2.3). As the Mₜ (base pair size) of each template was known (see Table 2.2), this allowed the determination of the number of moles of each template (moles = mass / Mₜ). The actual total number of copies of each template generated was then calculated using Avogadro's number (number of copies = moles of template x Avogadro's number [6.0225x10²³]). A series of stock solutions (using 1:10 serial dilutions) containing known template copy numbers (6.8 x 10⁸ - 6.8 x 10⁵ copies) was then made and then utilized as described in Section 2.5.4. The expected base pair sizes of the amplified template and sample products are given in Table 2.2.
Figure 2.2: Synthetic RNA template construction and preparation for QCRT-PCR

Complimentary sequences to 5', 3' and internal PCR primers for each template message

7 PCR cycles

Addition of flanking oligonucleotides

20 PCR cycles

Separation of products

Band excision

Upstream (5') amplification oligo sequences

Internal detection oligo sequences

Downstream (3') amplification oligo sequences

Adapted from Tarnuzzer et al, 1996
2.5.4 QCRT-PCR Protocol for mRNA Analysis

cDNA was synthesized in 50 μl volumes containing 0.1-1 μg of total RNA and dilutions of one, two or three competitive template RNAs in 10-fold serial dilutions from 6.8 x 10^8 - 6.8 x 10^2 copies/reaction, 2.5 mM oligo(dT)_16 (Gibco), 11.5 mM MgCl₂ (Perkin-Elmer), 200 μM dNTP (Promega), 50 U/ml human placental ribonuclease inhibitor (Gibco), 10 mM Tris-HCl, pH 8.3 / 50 mM KC1 (Perkin-Elmer), and 200 U/μg RNA MMLV -RT (Gibco). The RT reaction was then performed for one cycle at 25°C for 10 minutes, one cycle at 42°C for 60 minutes and a final cycle at 94°C for 5 minutes (see Figure 2.3) in a thermocycler (Omnigene).

cDNA amplification by PCR (see Figure 2.3) was carried out in a 50μl reaction volume containing 5μl of the RT reaction, 200μM dNTP (Promega), 50 pmoles of each 3' and 5' PCR primer (Cruachem), 1.5 mM MgCl₂, 10 mM Tris-HCl, pH 8.3, 50 mM KC1, and 2.25U/reaction of Taq DNA polymerase (Perkin-Elmer). Amplification reactions were carried out in 1 cycle of 92°C for 5 minutes, 58°C for 2 minutes and 72°C for 3 minutes, followed by 40 sequential cycles of 92°C for 1.5 min, 58°C for 2 min, and 72°C for 3min, and a final extension cycle of 72°C for 10 minutes in a thermocycler (Omnigene).

2.5.5 Detection and Quantitation of QCRT-PCR Products

PCR products were separated for 90 minutes at 100V on 2% (wt/vol) agarose gels dissolved in tris-acetate (0.04M)/EDTA (0.001M) buffer (pH 8) containing 25ng/ml ethidium bromide, illuminated with UV light and photographed onto Polaroid film (Sigma). Photographs were then scanned into an IBM compatible computer (Hewlet-Packard Scanjet 4C, Deskscan version 2.3; Hewlet-Packard) and overall band
intensities (area x intensity) were measured using image analysis software (Bioscan Optimas; DataCell). Band intensity values were then normalized based on the molecular weight of the products. The log of the ratio of the band intensities of amplified sample cDNA to amplified synthetic template cDNA within each lane were plotted against the log of the copy number of the template added to the initial reaction. The quantity of the initial sample messages were determined where the ratio of the template and sample band intensities were equal to one (see Figure 2.3). As the total RNA and cell numbers for each sample were known (see Sections 2.3 and 2.6.2), this allowed expression of the mRNA copy number/10^3 cells.
Figure 2.3: Schematic representation of QCRT-PCR technique

**Key**

- **Synthetic Template**
  (can be any of templates 1 - 4, or a combination; see Figure 2.1)

- **Constant amount of sample RNA**
  (0.1-1μg of total RNA isolated from cultures, collagen lattices or tissues)

- **Specific 5' and 3' primer pairs**

  Specific primer pairs for the message of interest are added prior to amplification by PCR. These primer pairs can be for any of the messages from the templates shown in Figure 2.1. A maximum of ten messages can be analysed from one RT reaction.
**ISOLATION OF TOTAL RNA**

Constant amount of sample RNA

Synthetic template copy number

Final reaction volume (50µl) made up with dNTPs, RT, RNAse inhibitor and buffer

**RT REACTION**

Final reaction volume (50µl) made up with dNTPs, RT, RNAse inhibitor and buffer

Addition of primer pairs

e.g.,
e.g.,
e.g.,
e.g., etc.

**PCR**

One cycle

Forty cycles

One cycle

Product separation by gel electrophoresis

Image analysis, calculation of ratio of band intensities and determination of mRNA copy number
2.6 Quantitation of Growth Factor, Extracellular Matrix and Growth Factor Receptor Proteins

2.6.1 Conditioned Medium Production

Human Tenon's capsule fibroblasts were seeded into 150cm² tissue culture flasks (Marathon) at a density of 10,000 cells/cm² and allowed to settle overnight. The growth medium was aspirated, and the monolayers exposed to the antimetabolites 5FU (0.25, 2.5 and 25mg/ml), MMC (0.001, 0.01 and 0.1mg/ml) or PBS as described in Section 2.2. At days 3, 7, 14, 24, 36 and 48 post-exposure to antimetabolites, the monolayers were washed with PBS (5 changes of 50ml/wash), followed by the addition of 20ml of DMEM containing 1% (wt/vol) bovine serum albumin (BSA) and incubation at 37°C twice (4 hours each incubation) to ensure residual serum depletion. The monolayers were then incubated for 24 hours in 20ml of DMEM containing 1% (wt/vol) BSA at 37°C. BSA was used in culture medium to reduce the non-specific binding of cellular derived soluble factors, such as ECM and growth factors many of which are adhesive molecules, to the tissue culture flasks. This conditioned medium was then collected, filter sterilised through a 0.2µm filter (Sartorius) to remove debris and insoluble material and aliquotted (350µl) using siliconised micropipette tips into siliconised tubes and stored at -70°C until required.

2.6.2 Cell Number Determinations

Following the collection of conditioned medium described above, the remaining cell monolayers were removed from the flask bottoms by the addition of 10ml/flask of 0.15% (vol/vol) phosphate buffered trypsin/ 0.02% (wt/vol) EDTA solution (Gibco) and incubation for 3 minutes at 37°C. The resultant cell suspensions were transferred to sterile
centrifuge tubes and the enzymic activity of the solution neutralised with 10ml of DMEM/10% (vol/vol) NCS. Cells were then pelleted by centrifugation for 8 minutes at 200g, the supernatant discarded and the cells resuspended in 1.1ml of NCS. A 0.1ml aliquot of this suspension was then added to 20ml of Isoton II (Coulter Electronics) and counted for cell number, in triplicate, using a Coulter Counter (Model ZF). Total cell numbers were calculated and expressed as the mean of triplicate counts (+/- S.E.). The percentage of viable cells within each sample were also determined, using 0.04% (wt/vol) trypan blue (Sigma) in PBS for a dye exclusion test. Cell count data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) at individual time points using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.

2.6.3 Enzyme Linked Immunosorbent Assays (ELISAs)

Growth factors have been shown to play a central role in the process of wound healing, as described in Section 1, and so the production of these factors was investigated. Several methods have been used to identify and quantitate growth factor production including Western blotting, radioimmunoassay (RIA) and ELISAs. The method chosen for this study was ELISA as unlike Western blotting quantitative data could be obtained and the potential hazards associated with radioactive isotopes (^{125}I) in RIA could be avoided.

The growth factors chosen for study were TGF\(^{\beta}\), bFGF and EGF as not only are they examples of distinct growth factor families, but also have been directly linked with the
wound healing process (see Section 1). Commercially available ELISA kits (R and D Systems) were used for the quantitation of the growth factors TGFβ₁, bFGF and EGF in samples of conditioned medium from growth arrested monolayers days 3, 7, 14, 24, 36 and 48 post treatment (see Section 2.6.1). In order to reduce the possibility of inter-assay variation, all samples were analysed for each factor during the same ELISA experiment and results compared at the same dilution.

The levels of immunoreactive TGFβ₁ species in samples of conditioned medium were quantitated as described in the assay manufacturers instructions. As TGFβ₁ is usually present in its latent form, TGFβ₁ in samples of conditioned media was initially activated by the addition of 1M HCl (0.2ml HCl / 1ml of conditioned medium) and incubation for 10 minutes. The samples were then neutralised by the addition of 0.2ml of 1.2M NaOH / 0.5M HEPES. TGFβ₁ standards (0-2000pg/ml) and samples of acid-activated conditioned medium (200µl/well; neat) were added in triplicate to a 96 well microtitre plate that had been coated with recombinant human TGFβ type II soluble receptor. After a 2 hour incubation period at room temperature, the plate was washed with 400µl/well of a buffered surfactant solution (wash buffer) a total of three times. A polyclonal anti-human TGFβ₁ antibody conjugated to horseradish peroxidase was then added (200µl/well), and incubated for 1.5 hours at room temperature. The plate was then washed as described above and 200µl/well of substrate solution (equal volumes of a stabilized hydrogen peroxide solution and a stabilized tetramethylbenzidine solution) added, and incubated for 20 minutes at room temperature. The immunoreactions were stopped by the addition of 50µl/well of 2M sulphuric acid. The optical density of each well was determined at 450nm using a
microplate reader (Titertek Plus MS2). Standard TGFβ1 curve results were expressed as the means of triplicate wells (+/- S.E.), and an equation for this standard curve determined using a polynomial trendline fit generated using computer software (Microsoft Excel). The levels of TGFβ1 in triplicate conditioned medium samples were calculated from this standard curve equation and expressed as the mean (+/- S.E.). As the dilution of the conditioned medium sample and the number of cells these samples were generated from were known (see Section 2.6.2), this allowed the results to be expressed as the quantity of TGFβ1 produced (pg) / 10^5 cells. The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance. The manufacturers of this ELISA kit have demonstrated no cross reactivity with 69 other growth factors and cytokines apart from TGFβ1,2 (17%), intra-assay coefficient of variance to be 5.2-7.5% and the inter-assay coefficient of variance to be 3.2-6.3%.

For the quantitation of immunoreactive bFGF species in conditioned medium samples, the assay procedure below was followed. Prior to the addition of standards or samples, 50µl/well of assay diluent RD1J (buffered protein base for culture media testing) was added. The bFGF standards (0-320pg/ml) and conditioned medium samples (neat) were added to triplicate wells (200µl/well) of a 96 well microtitre plate that had been coated with a mouse anti-human recombinant bFGF antibody. The plate was then incubated for 2 hours at room temperature, followed by a total of three washes of
400µl/well with a buffered surfactant solution (wash buffer). A horseradish peroxidase conjugated polyclonal anti-human recombinant bFGF antibody was then added (200µl/well) and incubated for 2 hours at room temperature. The plate was then washed as described above, and 200µl/well of substrate solution (equal volumes of a stabilized hydrogen peroxide solution and a stabilized tetramethylbenzidine solution) were added and incubated for 20 minutes at room temperature. The development of the plate was stopped by the addition of 50µl/well of 2M sulphuric acid, and the optical densities of each well determined at 450nm using a microplate reader (Titertek Plus MS2). Standard bFGF curve results were expressed as the means of triplicate wells (+/- S.E.), and an equation for this standard curve determined using a polynomial trendline fit generated using computer software (Microsoft Excel). The levels of bFGF in triplicate conditioned medium samples were calculated from this standard curve equation and expressed as the mean (+/- S.E.).

As the dilution of the conditioned medium sample and the number of cells these samples were generated from were known (see Section 2.6.2), this allowed the results to be expressed as the quantity of bFGF produced (pg) / 10⁵ cells. Data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance. The kit manufacturers have determined the intra and inter-assay coefficients of variance as 3.3-5.7% and 7.7-9.4% respectively, and that the kit shows no significant cross-reactivity with 48 different cytokines including TGFβ and EGF.
Immunoreactive EGF was quantitated in samples of conditioned media collected as described in Section 2.6.1, using a specific ELISA. Briefly, 200μl/well of EGF standards (recombinant human EGF diluted in calibrator diluent RD5E at concentrations from 0-250pg/ml) and conditioned medium samples (neat) were added, in triplicate, to a 96 well plate that had been coated with a monoclonal anti-human EGF antibody. The plate was then incubated for 2 hours, aspirated and then washed a total of three times with 400μl/well of a buffered surfactant solution (wash solution). A polyclonal anti-human EGF horseradish peroxidase conjugated antibody was then added to each well (200μl/well) and the plate incubated for 1 hour. The plate was then washed a total of three times, as described above, prior to the addition of 200μl/well of a substrate solution (stabilized hydrogen peroxide / stabilized tetramethylbenzidine) and incubation for 20 minutes. Development of the plate was stopped by the addition of 50μl/well of 2M sulphuric acid (stop solution) and the optical densities of each well determined as described above for TGFβ1 and bFGF. Quantitation of immunoreactive EGF in samples of conditioned medium was also determined and analysed as described above for TGFβ1 and bFGF. The kit manufacturers have determined the intra and inter-assay coefficients of variance to be 2.6 - 3.2% and 5 - 5.8% respectively, and shown that there was no significant cross-reactivity with 38 different cytokines.

The production of a variety of ECM molecules has been implicated in the wound healing process (see Section 1). The molecules investigated in this study were collagen type I, collagen type III and fibronectin as they have been shown to be produced by fibroblasts and implicated as playing major roles in tissue repair following injury. The
analysis of these different ECM molecules in samples of conditioned medium were run at
the same time for each molecule to reduce the possibility of inter-assay variations, and
comparisons of quantitative data were made at the same sample dilutions for each
individual molecule.

The levels of collagen types I and III produced by growth arrested fibroblasts in
samples of conditioned medium, collected as described in Section 2.6.1, were determined
using direct ELISAs. This conditioned medium differed from that described in Section
2.6.1 in that it did not contain BSA. The reason for this was that as a direct ELISA
method was employed, where the protein of interest is bound directly to the ELISA plate,
binding of BSA may have reduced the quantity of collagen types I or III able to bind to the
ELISA plate. The methods for both of the collagen ELISAs are illustrated in Figure 2.4,
and only differed in the antigens used for the standard curves and the primary anti-collagen
antibody. The collagen ELISAs used in this study were developed by Dr. Peter Constable
(MD student; Wound Healing Group, Institute of Ophthalmology, London).

Collagen type I or collagen type III standards (EuroGenetics; diluted in PBS at
concentrations ranging from 0-10µg/100µl) or 100µl/well of conditioned medium and
bound to the plates at 4°C overnight. Conditioned medium samples were run at dilutions
of neat, 1:2, 1:8, 1:32 and 1:128, with results being compared only from the same
dilutions. Non-specific binding sites were then blocked by the addition of 300µl/well of
1% (wt/vol) BSA in PBS and incubation for 1 hour at room temperature. The plates were
then aspirated and the primary polyclonal antibody (goat anti-human collagen type I or III;
EuroGenetics) was added at a pre-determined optimal dilution of 1:2000 (100µl/well) and
incubated for 1 hour at room temperature. The plates were then aspirated and washed a
total of three times with PBS/1% (wt/vol) BSA (300μl/well). A horseradish-peroxidase
coujugated rabbit anti-sheep antibody (EuroGenetics) was then added (100μl/well) at a
pre-determined optimal dilution of 1:8000, followed by incubation for 1 hour at room
temperature. The plates were then washed a total of three times with PBS (300μl/well),
prior to the addition of 100μl/well of OPD substrate (SigmaFast tablets; Sigma). Plates
were then developed for a predetermined optimal period of 30 minutes at room
temperature, the reaction stopped by the addition of 50μl/well of 3M hydrochloric acid
and the optical density of each well determined using a Titertek Plus MS2 microplate
reader at a wavelength of 450nm. Standard collagen types I and III curve results were
expressed as the means of triplicate wells (+/- S.E.), and an equation for this standard
curve determined using a polynomial trendline fit generated using computer software
(Microsoft Excel). The levels of collagen types I and III in triplicate conditioned medium
samples were calculated from this standard curve equation and expressed as the mean
(+/- S.E.). As the dilution of the conditioned medium sample and the number of cells these
samples were generated from were known (see Section 2.6.2), this allowed the results to
be expressed as the quantity of collagen types I or III produced (ng) / 10^6 cells. The data
was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using
a one-way analysis of variance (ANOVA). The observed significance levels from multiple
comparisons were adjusted using the Bonferroni test. A probability (p) value of less than
0.05 (p<0.05) was considered as indicating significance.

Although this assay showed no cross-reactivity with fibronectin, there was
cross-reactivity of the anti-collagen type I antibody with purified collagen type III and vice
versa. The cross-reactivity of these antibodies / antigens were taken into account for data
analysis (see Section 3).
The levels of fibronectin produced by growth arrested fibroblasts were quantitated using a double sandwich ELISA described by Khaw (PhD thesis, University of London, 1994). Briefly, conditioned medium samples were collected as described in Section 2.6.1, from cultures growth arrested as described in Section 2.2. The ELISA technique used was similar to that illustrated in Figure 2.4 for collagen types I and III, the major differences being that two monoclonal anti-human fibronectin antibodies raised to different antigenic sites were used instead of one, and that one of these antibodies was conjugated to horseradish peroxidase. Firstly, 96 well microtitre plates were coated at 4°C overnight with 100µl/well of a monoclonal anti-human fibronectin antibody (Dako; A245) diluted 1:1000 in PBS. The wells were then aspirated, and excess protein binding sites blocked by the addition of 300µl/well of 1% (wt/vol) BSA in PBS and incubation for 1 hour at room temperature. Plates were then aspirated and 100µl/well of fibronectin standards (0-10µg/ml), or samples of conditioned medium from growth arrested fibroblasts diluted in 1% (wt/vol) BSA were added, followed by incubation for 2 hours at room temperature. As there was no way of knowing what levels of fibronectin were present in conditioned medium samples prior to the ELISA, samples were assayed at dilutions of neat, 1:10, 1:20, 1:40, 1:80 and 1:100. In order to reduce the variability of results, all samples were run at the same time and results only compared from the same dilution. The wells were then aspirated and washed a total of three times with PBS/1% (wt/vol) BSA (300µl/well). The second monoclonal antibody (Dako; P246), which was horseradish peroxidase labelled, was then added to the plates (100µl/well) at a dilution of 1:4000 in PBS/1% (wt/vol) BSA, followed by incubation for 1 hour at room temperature. The plates were then washed a total of three times with PBS (300µl/well), prior to the addition of 100µl/well of OPD
substrate (see above). Plates were then developed for 45 minutes at room temperature, the reactions stopped by the addition of 50μl/well of 3M hydrochloric acid and the optical density of each well determined using a Titertek Plus MS2 microplate reader. Standard fibronectin curve results were expressed as the means of triplicate wells (+/- S.E.), and an equation for this standard curve determined using a polynomial trendline fit generated using computer software (Microsoft Excel). The levels of fibronectin in triplicate conditioned medium samples were calculated from this standard curve equation and expressed as the mean (+/- S.E.). As the dilution of the conditioned medium sample and the number of cells these samples were generated from were known (see Section 2.6.2), this allowed the results to be expressed as the quantity of fibronectin produced (μg) / 10^5 cells. The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.
Figure 2.4: Schematic representation of an enzyme linked immunosorbent assay (ELISA) for collagen

Binding of antigen to plate.

Blocking of non-specific binding sites with BSA.

Addition of primary antibody

Wash

Addition of horseradish peroxidase conjugated antibody

Wash

Addition of substrate (hydrogen peroxide) and chromogen (OPD), development and determination of optical density at 450nm
2.6.4 Radioreceptor Assays (RRAs)

As outlined in Section 1, the wound healing process is under the control of several growth factors and the receptors through which they elicit their effects. The growth factor families that have been implicated in this healing process include the TGFβ, FGF, EGF, IGF and PDGF families. As for growth factor production, the receptor of three members of these families were investigated in this study: the TGFβ type II receptor, the bFGF and the EGF receptors.

The method used to quantitate cell surface receptor numbers was that described by Massague (Massague, 1987). Briefly, human Tenon's capsule fibroblasts were seeded at a density of 10 000 / cm² into 48 well tissue culture plates and allowed to settle overnight. The medium was then aspirated from each well and the cells exposed in sextuplicate to 5FU (0.25, 2.5 and 25mg/ml), MMC (0.001, 0.01 and 0.1mg/ml) or PBS as controls (see Section 2.2). The wells were then washed five times in total with PBS (500μl/well each wash) and exposed to serum containing medium. The numbers of growth factor receptors were then quantitated days 7, 24 and 48 post antimetabolite exposure as described below.

Plates were washed with PBS (5 x 500μl/well) prior to incubation in serum free medium for 24 hours (500μl/well). Each well was then aspirated and either counted for cell numbers (triplicate wells) or had 50μl/well of binding buffer (DMEM/0.1% (wt/vol) BSA and 25mM HEPES, pH 7.4) added. Varying concentrations (0, 10⁻¹²M, 10⁻¹¹M, 10⁻¹⁰M, 10⁻⁹M, 10⁻⁸M and 10⁻⁷M) of each of the unlabelled growth factors TGF-β, bFGF or EGF (Sigma) were added to triplicate wells in a volume of 100μl. To each well 100μl of a [¹²⁵I]-labelled growth factor (all Amersham) was then added (100000 cpm/well), and the plates incubated at 4°C overnight to avoid receptor internalisation. The plates were
then aspirated and each well washed a total of four times (500μl/well) with binding buffer (DMEM/0.1% (wt/vol) BSA and 25mM HEPES, pH 7.4). Cell monolayers were then solubilised by the addition of 500μl/well of solubilisation buffer (1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol and 0.01% (wt/vol) BSA, pH 7.4). Solubilisation of the cells was then checked microscopically, prior to counting 450μl of each extract using a gamma counter (Wilj International Ltd.). Receptor numbers were then calculated via Scatchard analysis. The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.
2.7 Fibroblast Settlement and Migration

2.7.1 Migration Assay

The ability of fibroblasts to migrate is an important factor in the wound healing process. As illustrated in Section 1, there are several assays which have been used to study migration. Initially, modified forty-eight microwell two tiered blindwell migration chambers (NeuroProbe) based upon the original chambers described by Boyden (Boyden, 1962) were used to study migration. Although these chambers allow the analysis and comparison of multiple samples (chemoattractants) utilising small volumes, some technical difficulties were encountered. These included the introduction of air bubbles into upper (containing cells) and lower (containing chemoattractant) wells during pipetting. This invariably resulted in the inability of cells to migrate. As a result, transwell tissue culture inserts (Costar) for 24 well plates were used as they were found to be easier to use although employing larger volumes of chemoattractant. This migration assay is illustrated in Figure 2.5 and described below.

Preliminary experiments performed to standardise the migration assay included chequerboard analysis (Zigmond and Hirsch, 1973) to determine if the migratory response to FCS was primarily chemotactic or chemokinetic, in addition to determining the optimal concentration of chemoattractant. This was found to be 20% (vol/vol) FCS in the lower chamber. The optimal length of migration assay was also determined and found to be 16 hours. As a result, the following experiments were performed using 20% (vol/vol) FCS as the chemoattractant over a migration assay time of 16 hours (see Section 3).

Human Tenon's capsule fibroblasts were seeded at a density of 10 000 cells/cm² in 6 well tissue culture plates (Marathon), and allowed to settle overnight. The overlying medium was the aspirated and monolayers exposed to antimetabolites (0.25, 2.5 and
25mg/ml 5FU; 0.001, 0.01 and 0.1mg/ml MMC or PBS) as described above in Section 2.2. The growth medium was changed on these cultures every 3 to 4 days. Cell monolayers were then harvested by trypsinisation days 3, 7, 14, 24, 36 and 48 post treatment and total viable cell numbers determined on a haemocytometer using a trypan blue dye exclusion test. Either 600μl of serum-free medium (control) or medium containing 20% (vol/vol) foetal calf serum (FCS; Gibco) was added to triplicate wells of a 24 well plate (Marathon). Transwell tissue culture inserts (Costar) were then placed into each well (Figure 2.4) and 100μl of serum-free medium containing 8 000 viable cells from each growth arrested sample were added to the top chamber. Cells were allowed to settle and migrate through the polycarbonate membrane in the transwell (8μm pores) over a period of 16 hours at 37°C in 5% CO₂ in air. After this migration period, medium was flicked off the transwells, which were then washed in PBS followed by fixation in 400μl of 100% ethanol (5 minutes). The fixative was then removed and each transwell was then immersed in haematoxylin (BDH) for 5 minutes, excess dye being removed and blueing of haematoxylin being achieved by rinsing the transwell in tap water. For settled cell number determinations, the bottom of the membrane of the transwell (migrated side) was cleared of cells using a cotton wool swab. For migrated cell number, the top of the transwell membranes were cleared of cells. The membranes were then cut out of the transwells using a scalpel blade, and mounted on glass slides with an aqueous mountant (Dako). Sets of triplicate membranes were then counted for total cell numbers settled, migrated to chemoattractant (chemotaxis) or migrated to serum-free medium (chemokinesis). For settled cell numbers, due to the high seeding density (8000 cells) per well, ten high power fields (magnification X 400) were counted on each of the triplicate membranes. These results were then pooled and analysed as described below. For migrated cells, the whole of
the membrane was counted as the maximal number of cells was never particularly high (usually between 300-400 cells per membrane). Results were expressed as the mean percentage of cells (settled or migrated; +/- S.E.) compared to the PBS treated controls (which were taken as 100% migration). The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.
Figure 2.5: Schematic representation of migration assay

1. Addition of chemoattractant or serum-free medium (control)

2. Addition of transwell containing cells in serum-free medium

3. Settlement and migration of cells

4. Fixation and staining of cells

5. Migrated (or settled) cell numbers counted
2.8 Extracellular Matrix Contraction

The contraction of the extracellular matrix is a crucial component of the wound healing response and, as shown in Section 1, has been studied using a variety of \textit{in vitro} models including silicon sheets and collagen lattices.

2.8.1 Silicon Sheet Contraction Assay

Ultrathin silicon sheets were prepared as described by Harris \textit{et al} (1981). A 2cm\(^2\) glass coverslip (BDH) was covered with a drop of 60000 centistoke dimethylpolysiloxane (Sigma), and the surface passed through a bunsen burner flame (approximately 2 seconds). This resulted in the formation of a skin of cross-linked silicone rubber, which was situated on top of a fluid silicone phase. The coverslip was then placed into a 8cm\(^2\) petri dish and covered with 3ml of DMEM/10\% (vol/vol) NCS containing 5000 cells. Cells were allowed to settle overnight prior to observation by phase contrast microscopy. Although this model of contraction showed quite clearly the tractional forces generated by cells migrating upon their substratum, the actual method itself was not particularly reproducible. The collagen contraction assay described below was used as it was a far more reproducible, and a better \textit{in vivo} representative, method for investigating ECM contraction.

2.8.2 Collagen Contraction Assay

The use of an \textit{in vitro} model for quantitating the contraction of a collagen matrix by cells entrapped within this matrix was originally reported by Bell \textit{et al} (1979). This model has since been used extensively, as have a number of variations, for investigating the process of collagen contraction as well as the behaviour of cells within extracellular matrix components (see Section 1). The model used in this study was the same as that described
by Bell et al (1979), where fibroblasts are seeded into collagen type I free floating lattices. The formation of cell-populated collagen matrices involved the mixing of a cell suspension with solubilised collagen and culture medium. The solution was then rapidly polymerised by raising the solution pH and temperature, trapping the cells within a three-dimensional collagenous matrix.

2.8.3 Preparation and Casting of Collagen Lattices

Collagen lattices were prepared by adding 0.6ml of 5mg/ml rat tail type I collagen (Sigma) dissolved in 0.1% (vol/vol) glacial acetic acid (Fisons) to 0.35ml of concentrated medium stock solution (15ml of 10X DMEM; Gibco, 35ml distilled water, 1.5ml of 2mM L-glutamine, 1.5ml of 250μg/ml fungizone, 1.5ml of 10000U/ml penicillin/10000μg/ml streptomycin solution and 4ml of 7.5% (wt/vol) sodium bicarbonate in sterile distilled water. This solution was rapidly mixed and the pH raised to 7.4 by the dropwise addition, with mixing, of 0.1M NaOH. Fixed cell numbers in a volume of 0.15ml NCS were then added and again the solution was rapidly mixed to disperse the cells. Aliquots of this collagen/medium/cell mixture were then pipetted into 6 well (1ml/well), 24 well (250μl/well) or 48 well plates (125μl/well), and the plates rotated so that the mixture evenly covered the bottom of each well. The plates were then incubated at 37°C to polymerise the collagen lattices rapidly (within 2-3 minutes), entrapping the cells throughout the three-dimensional matrix. The lattices were then overlaid with DMEM/10% (vol/vol) NCS and carefully released from the bottom of the wells with a micropipette tip, ensuring that the lattices floated freely in the growth medium.
Collagen contraction has been shown to be dependent upon collagen concentration (see Section 1). A optimal collagen concentration of 5mg/ml was determined by pilot studies, this concentration allowing a measurable contraction rate. Also commercially prepared collagen (Sigma) was chosen for use in experiments as this eliminated the batch to batch variation of collagen prepared in the laboratory (Dr. Ank Mazure, personal communication). In all initial experiments, collagen lattices were seeded into 8cm² culture dishes. However, due to the large cost of the collagen used smaller well plates were employed (24 well and finally 48 well) for the remainder of the study. In order to allow direct comparisons between these early and later experiments, the initial format of expressing maximal collagen lattice area at 8cm² was followed throughout, as control lattices were found to contract to the same degree in standardisation experiments regardless of the plate format used.

2.8.4 Determination of Optimal Collagen Lattice Cell Number

In order to determine the number of cells required to give a measurable degree of contraction during the 7 day assay, triplicate collagen lattices were prepared as described in Section 2.8.3, and seeded into 6 well plates containing 50 000, 100 000, 250 000 or 500 000 cells/ml of lattice. The lattice areas were measured days 1, 3 and 7 post seeding as described in Section 2.8.5, and expressed as the mean (+/- S.E.). The growth medium was changed on all lattices day 3 post seeding.

2.8.5 Measurement of Collagen Lattice Area

At specific time points post seeding, plates containing collagen lattices were placed on an acetate sheet of known area (275.25cm²) and illuminated from below with a light
box. Photographs of the lattices and calibration grid were then taken. The calibration grid and lattice images were then digitised directly from the photographs using a Kurta 1212 Summasketch Tablet board (Kurta) into an IBM compatible computer and analysed using SigmaScan software (Jandel Scientific). Lattice areas were expressed as the means of triplicate lattices (+/- S.E.). This method of measuring lattice area was used successfully for 6 and 24 well plates. However, due to the small size of 48 well plates a different method of lattice image capture was employed. This involved the use of a 8mm camcorder equipped with a macro-lens and viewfinder (Sharp). Briefly, at specific time points post-seeding plates containing lattices were illuminated from below with a light box. Images of a control well of known area (i.e., an area calibration grid) as well as lattices in individual wells were traced manually onto acetate sheets directly from the viewfinder. These images were then digitised and analysed as described above for 6 and 24 well plates.

2.9 Collagen Contraction, Cellular Activation, Cell Viability and Number. Cytoskeleton and Morphology in Collagen Lattices

2.9.1 Preparation of Activated and Nonactivated Fibroblast Cultures

It has been suggested that a more aggressive healing response may occur in vivo as a result of increased preoperative cellular activation (Khaw et al. 1994a). Therefore, cultures of activated fibroblasts were prepared by growing human Tenon's capsule fibroblasts to near confluence in the presence of 10% (vol/vol) NCS. Cultures of nonactivated fibroblasts were prepared by growing the cells to near confluence in the presence of serum, washing the monolayers three times with PBS (50ml each wash), and culturing the cells in serum-free medium for 60 hours at 37° C in 5% humidified CO₂ in air.
to ensure residual serum depletion. The viability of all nonactivated fibroblasts was confirmed using trypan blue exclusion staining. These cultures were then used to seed collagen lattices.

To partially characterize these cells, cell monolayers were initially immunostained for Ki67 (supplier of antibody - The Binding Site). Briefly, monolayers were firstly fixed in methanol (Fisons) at -18°C for 30 minutes prior to being rehydrated in PBS and microwaved to unmask antigenic sites (Cattoretti et al. 1992). The monolayers were then immunostained using a biotin-streptavidin peroxidase technique (Geudson et al. 1979) with diaminobenzidine as the chromogen. Ki67 is a specific marker of metabolically active proliferating cells (those in G1, G2, S or M phases of the cell cycle; Cattoretti et al. 1992). Additionally, cells in tissue culture flasks were fixed, processed and viewed by scanning electron microscopy (SEM; see Section 2.9.8) for the presence of cell surface processes as these have been reported as being present on activated cells (Alexander et al. 1989).

2.9.2 Exposure of Cells in Collagen Lattices to Antimetabolites

Triplicate lattices seeded with 500 000 cells/ml of lattice were prepared in 6 well plates as described in Section 2.8.3. The overlying medium was removed and the lattices exposed to the antimetabolites 5FU (0.01-25mg/ml), MMC (0.01-1mg/ml) and PBS or water as controls. The growth arresting treatment was removed after 5 minutes incubation at 37°C and the lattices washed five times with PBS (5ml/wash). Lattices were then overlaid with 3ml of fresh growth medium containing 10% NCS. Lattice area measurements (see Section 2.7.5) were made days 1, 3 and 7 post seeding. The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) individual
using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.

2.9.3 Lattice Cell Number and Cell Viability Determinations

Lattices were harvested immediately and days 1, 3 and 7 post exposure to antimetabolites, PBS or water and washed with PBS (3x3ml), and then immersed into a 1ml solution of 4mg/ml collagenase type IV (Sigma) in PBS (Ehrlich and Wyler, 1983) at 37°C for 30 minutes. The cell suspension was then centrifuged for 8 minutes at 200g and the resultant pellet containing cells freed from the collagen was resuspended in 0.04% (wt/vol) trypan blue (Sigma) in PBS. Cell suspensions from each of the triplicate lattices were then counted three times each, on a haemocytometer, for total cell number and non-viable cells which stained blue. The total and non-viable cell numbers were expressed as the mean percentage of the cells immediately following treatment and as the mean percentage of total cells respectively. Cell number and viability data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.

2.9.4 Immunofluorescent Staining of the Actin Cytoskeleton of Cells Populating Collagen Lattices

Whole lattices containing cells were probed for filamentous actin days 1, 3 and 7 post seeding. Briefly, lattices were washed three times with PBS (3ml/wash) and fixed in
10% (vol/vol) formal saline for 1 hour at room temperature. Lattices were then rinsed three times (20 minutes/rinse) in tris-buffed saline (TBS; 25 mM Tris, 140 mM NaCl, 3 mM KCl, pH 7.4) prior to an overnight incubation in fluorescein isothiocyanate (FITC)-phalloidin (Sigma) at 2.5 μg/ml in TBS. Lattices were then rinsed three times in TBS (20 minutes/rinse) followed by an overnight incubation in 7-aminoactinomycin D (Sigma) at 100 μg/ml in PBS. The lattices were then rinsed and mounted in PBS and viewed by fluorescence microscopy (Zeiss Axiphot; Carl Zeiss). Double labelled photographs were taken of the cells by firstly photographing the cytoskeleton (for FITC label) at an excitation/emission wavelength of 490/525 nm, and then photographing the cell nuclei on the same frame at a tetra-rhodamine isothiocyanate (for 7-aminoactinomycin D label) excitation/emission wavelength of 535/580 nm.

2.9.5 Exposure to Antimetabolites and Long Term Collagen Contraction

Lattices populated with cells (5x10⁵/ml of lattice) were exposed in triplicate to antimetabolites as described in Section 2.9.2. Lattice areas were then measured days 3, 7, 14, 24, 36 and 48 post treatments, as described in Section 2.8.5. Growth medium was changed every 3 to 4 days and cell morphology was monitored throughout the culture period as described below.

2.9.6 Cellular Morphology Within Collagen Lattices by Phase Contrast Microscopy

Cells seeded within collagen lattices were frequently observed using a phase contrast microscope (Model IMT; Olympus). Documentation of cellular morphology throughout the process of collagen contraction was achieved using a camera (Olympus
C-35AD) mounted onto the microscope and connection to an automatic exposure unit (PM10-AD; Olympus).

### 2.9.7 Preparation of Collagen Lattices for Transmission Electron Microscopy (TEM)

Collagen matrices were washed in PBS (3 x 5ml) and then fixed in 2.5% (vol/vol) glutaraldehyde (EM grade vacuum distilled; Fisons) in Sorenson's phosphate buffer, pH 7.4. The specimens were then postfixed for 1 hour in 1% (wt/vol) osmium tetroxide in the same buffer, followed by a 15 minute wash in buffer alone. The specimens were then washed in three changes of distilled water (10 minutes each) before being left overnight at 4°C in a 0.5% aqueous solution of uranyl acetate (Agar Scientific). The specimens were then rinsed in distilled water followed by dehydration (15 minutes in each solution) through a graded series of alcohols (25%, 50%, 70%, 80%, 90%, and 3 times in 100%; Analar grade, BDH). The specimens were subsequently embedded in Araldite resin (Agar Scientific). This was performed with propylene oxide (PO) as the intermediate fluid. First 100% PO was used (two 15 minute changes), then a 75% PO/25% Araldite mix for two hours, followed by 50%/50% and 25%/75% also for two hours each. The specimens were left in 100% Araldite overnight for maximum infiltration, and then placed in a mould in fresh Araldite to polymerise in a 60°C oven for 24 hours. All blocks were allowed to cure for 1-2 days before cutting.

Ultrathin sections (50-70nm) were cut using the Ultracut S microtome (Leica). The sections were flattened with chloroform, placed on 200 mesh copper grids (0.05mm; Agar Scientific). Sections were stained with 1% (wt/vol) uranyl acetate for 15 minutes, rinsed in water and stained in 0.4% (wt/vol) Reynolds lead citrate for 6-7 minutes,
followed by another rinse in water. Specimens were viewed in a Jeol 1010 transmission electron microscope (Jeol UK) operating at 80kV.

2.9.8 Preparation of Collagen Lattices for Scanning Electron Microscopy (SEM)

Primary and secondary fixation were the same as for TEM. However, following the post osmication buffer wash, SEM specimens were washed three times in distilled water and dehydrated through a graded series of alcohols as described for TEM specimens. Following the final change of 100% alcohol, the specimens were critical point dried (Polaron), mounted on metal stubs with double sided adhesive tape and gold coated (approximately 12-16nm thickness) using a Polaron sputter coater. The three-dimensional matrices were examined in a Jeol 6100 scanning electron microscope (Jeol U.K) using secondary electron detection.

2.10 Matrix Metalloproteinases (MMPs) and the Tissue Inhibitors of Matrix Metalloproteinases (TIMPs) in Collagen Contraction

MMPs and TIMPs have been shown to be involved in the remodelling of ECM and the movement of a variety of cell types through ECM membrane constructs (see Section 1). As the contraction of collagen is regarded as resulting from the migration of cells within the collagen matrix and the subsequent generation of tractional forces upon their substratum, this study investigated the role of MMPs and TIMPs in fibroblast mediated collagen contraction.
2.10.1 Assays for MMPs and TIMPs

2.10.1.1 Extraction of Total RNA from Cells Populating Collagen Lattices

The overlying medium was aspirated from collagen lattices, followed by three washes in PBS (5ml/wash). Lattices were then partially dehydrated on grade 1 filter paper (Whatman) to remove excess fluid that may have resulted in dilution of the RLT lysis buffer used for RNA extraction. The lattices were then transferred to a tissue homogeniser (BDH/Merck) containing 3ml of RLT lysis buffer (see Section 2.3) and homogenised until the lattices were no longer visible. Samples were then passed through a 25 gauge needle attached to a 5ml syringe and subjected to the protocol described in Section 2.3.

2.10.1.2 QCRT-PCR Analysis of MMP and TIMP mRNA

Collagen lattices were seeded with 500 000 cells/ml of lattice in 6 well tissue culture plates and harvested for total RNA isolation as described in Section 2.10.1.1, at 9 hours, 1 day and 7 days post seeding (6 lattices per time point). Total RNA was also isolated from $3 \times 10^6$ cells in monolayer culture. Monolayer cultures were used as a comparable control for MMP and TIMP expression so that differences in quantites could be attributed to culture within and during contraction of collagen lattices. Cells seeded upon the surface of collagen lattices were also selected as controls to see if the production of MMPs and TIMPs was different depending if cells having three dimensional contact with collagen, compared to two dimensional contact with a collagen matrix. Fixed quantities of RNA (1μg) from each of these samples were analysed for MMP 1, MMP 2, MMP 3, MMP 9 and TIMP 1 mRNA expression using the protocols described in Sections 116.
2.5.4 and 2.5.5. Levels of MMP and TIMP mRNA were expressed as copy numbers/10^3 cells.

2.10.1.3 Collection of Conditioned Medium for Gelatin Zymography

Collagen lattices containing 500 000 cells/ml of lattice in 6 well plates or with 7.5x10^4 cells on their surface were prepared as described in Section 2.8.3, the only difference being that the NCS used within the lattices and in the growth medium had been depleted of MMPs by incubation with gelatin coated Sepharose 4CLB beads (Pharmacia; diluted 1:10 in NCS) for 2 hours at 4°C as described by Azzam et al (1992). Beads were then removed from NCS by filtration through a 0.2μM filter (Sartorius). Medium overlying the lattices (5ml) was collected days 1, 3 and 7 post-seeding and stored at -70°C prior to lyophilisation at -50°C. The samples were then reconstituted in 2ml of PBS and stored in aliquots at -70°C until required for MMP analysis by gelatin zymography (see Section 2.10.1.5). Cells in this collagen contraction model have been shown not to proliferate (see Section 1) and so it was assumed that the initial seeding cell number and the lattice cell number at the time of conditioned medium collection were the same.

2.10.1.4 Extraction of MMPs from Collagen Lattices for Gelatin Zymography

Collagen lattices were prepared as described in Section 2.8.3 above. Lattices containing cells were harvested days 1, 3 and 7 post seeding, washed with PBS (3x5ml), transferred to a tissue homogeniser (BDH/Merck) containing 1ml of 0.5% (vol/vol) Triton X-100 in PBS and homogenised. Samples were then centrifuged at 200g for 8 minutes to remove gross debris and the supernatant stored in aliquots at -70°C prior to MMP analysis by gelatin zymography (see Section 2.10.1.5 below).
2.10.1.5 Analysis of MMP Profiles by Gelatin Zymography

Samples of conditioned medium, normalised for cell numbers, from lattices with cells seeded within the matrix or seeded upon the matrix (15μl) for gelatin zymography (Heussen and Dowdle, 1980) were denatured with an equal volume of dissociating buffer (125mM Tris-HCl, pH 6.8, 5% (vol/vol) glycerol, 4% (wt/vol) sodium dodecyl sulphate, 0.005% (vol/vol) bromophenol blue; Novex, R & D Systems Ltd) for 10 minutes at room temperature. These samples and controls of growth medium containing gelatinase-depleted serum were then resolved in conjunction with prestained, broad range molecular weight standards (M, 7 200 - 208 000; Bio-Rad) on a 10% (vol/vol) tris glycine polyacrylamide gel containing 0.1% (wt/vol) gelatin (Novex) for 90 minutes at a constant voltage (125V) and current (40mA) in running buffer (25mM Tris base, 192mM glycine, 0.1% (wt/vol) sodium dodecyl sulphate, pH 8.3; Novex). The gels were then carefully removed and incubated in renaturing buffer (2.5% (vol/vol) Triton X-100; Novex) for 30 minutes at room temperature. The renaturing buffer was then removed and replaced with developing buffer (10mM Tris base, 40mM Tris-HCl, 200mM NaCl, 5mM CaCl₂, 0.02% (vol/vol) Brij35; Novex) for a further 30 minutes. Fresh developing buffer was then added and the gels incubated at 37°C for 20 hours prior to staining in 0.5% (wt/vol) Coomassie blue (Bio-Rad) in 45% (vol/vol) methanol /45% (vol/vol) distilled water 5% (vol/vol) glacial acetic acid on an orbital shaker for 2 hours. As Coomassie blue stains protein, MMP activity appeared as clear bands on a blue background where the gelatin in the gel had been degraded. In order to maximise band intensities, the gels were destained in 45% (vol/vol) methanol / 45% (vol/vol) distilled water/ 5% (vol/vol) glacial acetic acid until areas of activity were totally clear.
MMPs are initially produced as high molecular weight inactive proenzymes which are then processed to give rise to the active, smaller molecular weight form of the enzyme (Woessner, 1991; Matrisian, 1992). However, by zymography the inactive enzymes can be partially activated and therefore appear as areas of gelatinolytic activity. Prior to zymography, samples can be incubated with aminophenylmercuric acetate (APMA; Sigma) which causes a confirmational change to occur within the inactive proenzymes allowing autocatalytic cleavage to their active form (Stricklin et al. 1983). In order to obtain full MMP profiles (active and inactive proenzymes), samples were run with or without activation by incubation with 2mM APMA for 2 hours at 37°C. The molecular weights of proform and active MMP species were calculated by comparison to the prestained molecular weight standards.

Confirmation that the areas of activity were in fact MMPs was achieved as follows. Samples of day 7 conditioned medium were separated by gelatin zymography, incubated in renaturing solution and the first incubation in developing buffer as described above. The gels were then cut into single lane strips and incubated in increasing concentrations (0, 2nM, 200nM, 20μM and 2mM) of 1, 10-phenanthroline (Sigma) in developing buffer at 37°C for 20 hours prior to staining in 0.5% (wt/vol) Coomassie blue (Bio-Rad) in 45% (vol/vol) methanol /45% (vol/vol) distilled water 5% (vol/vol) glacial acetic acid on an orbital shaker for 2 hours. Phenanthroline is a zinc chelator and has previously been used in several studies to show that inhibition of gelatinolytic activity indicates that this activity is due to MMPs (Le et al. 1991; Galis et al. 1995).
2.10.1.6 Quantitation of MMPs and TIMPs by Enzyme Linked Immunosorbent Assays (ELISAs)

As for growth factors (Section 2.6.3), the use of ELISA kits for the quantitation of MMPs and TIMP 1 proteins was chosen in preference to Western blotting. For each ELISA, samples were assayed in one run in order to eliminate the possibility of inter-assay variation. Samples of conditioned medium were collected from contracting collagen lattices as described in Section 2.10.1.3 prior to quantitation of MMPs 1, 2, 3 and TIMP 1 using specific ELISAs. For all ELISAs, samples of conditioned medium were assayed at a range of dilutions (neat, 1:5 and 1:10) to try to ensure a reading that lay within the range of the respective standard curves. Calculation of the quantities of MMPs and TIMP 1 were only made from samples run at the same dilutions. TIMP 2 was not investigated as no quantitative assay was available for this protein at the time of study.

The levels of total MMP 1 (pro- and active) in samples of conditioned medium from contracting collagen lattices (days 1, 3 and 7 post seeding) were quantitated using a specific double sandwich ELISA kit (Amersham), according to the manufacturer's instructions. Briefly, MMP 1 standards (0 - 100ng/ml) and conditioned medium samples were added to triplicate wells (100μl/well) of a 96 well plate that had been coated with a mouse monoclonal anti-human MMP 1 antibody. Following incubation for 2 hours, the plate was washed with 400μl/well of wash buffer (0.0067M phosphate buffer, pH 7.5 containing 0.033% vol/vol Tween 20) a total of four times. A rabbit polyclonal anti-MMP 1 antibody was then added to each well (100μl) and incubated for 2 hours. The plate was then washed again, as described above, prior to the addition of 100μl/well of a donkey anti-rabbit horseradish peroxidase conjugated antibody and incubation for 1 hour. After
four more washes with buffer, 100µl/well of 3, 3', 5, 5'-tetramethylbenzidine (TMB)/hydrogen peroxide in dimethylformamide (20% vol/vol) was added and the plate incubated for 30 minutes. Immunoreactions were then stopped by the addition of 100µl/well of 1M sulphuric acid and the optical densities of each well determined at 450nm on a microplate reader (Titertek Plus MS2). Standard MMP 1 curve results were expressed as the means of triplicate wells (+/- S.E.), and an equation for this standard curve determined using a polynomial trendline fit generated using computer software (Microsoft Excel). The levels of MMP 1 in triplicate conditioned medium samples were calculated from this standard curve equation and expressed as the mean (+/- S.E.). As the dilution of the conditioned medium sample were known, this allowed the results to be expressed as the quantity of MMP 1 produced per unit volume (ng/ml). The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance. The manufacturers of this ELISA kit have demonstrated no significant cross reactivity with MMPs 2, 3 or 9, intra-assay coefficient of variance to be 5.5 - 7.9% and the inter-assay coefficient of variance to be 11.6 - 13.2%.

The quantities of proMMP 2 (and proMMP 2 complexed with TIMP 2) produced during collagen contraction were determined using a one-step sandwich enzyme immunoassay system (Fuji Chemicals; Fujimoto et al. 1993). MMP 2 standards (0 - 400ng/ml) and samples (50µl of each standard or sample) were pipetted in triplicate into borosilicate tubes (12 x 75mm, Corning). Conditioned medium samples were assayed at
the same dilutions described for MMP 1. A horseradish peroxidase conjugated monoclonal anti-human MMP 2 antibody was then added (300μl/tube), followed by a polystyrene bead (1 bead/tube) coated with a monoclonal anti-human MMP 2 antibody which had been raised to a different antigenic site than the enzyme conjugated MMP 2 antibody. All tubes were then incubated for 1 hour prior to four washes (3ml per tube/wash) with a sodium phosphate buffer (pH 7). The washed beads were then transferred to new tubes and 300μl/tube of a substrate solution (0.02% hydrogen peroxide / 0-phenylenediamine) added and incubated for 30 minutes. Immunoreactions were stopped by the addition of 1.5ml of 1.3M sulphuric acid, 100μl aliquots of each specimen transferred to a 96 well plate and the optical densities determined at 450nm on a microplate reader (Titertek Plus MS2). The levels of MMP 2 in samples of conditioned medium were calculated by comparison to the standard curve, as described above for MMP 1. The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) at individual time points using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance. The manufacturers have demonstrated that the inter-assay coefficient of variance for the kit is less than 12%.

ProMMP 3 (free and complexed with TIMP 1 or TIMP 2) levels produced during collagen contraction were determined using a specific one-step sandwich enzyme immunoassay (Fuji Chemicals; Obata et al. 1992). Triplicate samples of standards (12.5 - 400ng/ml) and conditioned medium from contracting collagen lattices (days 1, 3 and 7 post seeding, see Section 2.10.1.3) were pipetted into borosilicate tubes (20μl of
Conditioned medium samples were assayed at the same dilutions described for MMP 1. A horseradish peroxidase conjugated monoclonal anti-human antibody was then added (300μl/tube) followed by the addition of one polystyrene bead coated with a monoclonal anti-human MMP 3 antibody, raised to a different antigenic site than the peroxidase conjugated antibody, to each tube. Samples were then incubated for 90 minutes, and immunoreactions stopped by the addition of 500μl/tube of wash solution (sodium phosphate buffer, pH 7). The wash solution was aspirated and the tubes washed a further four times with 4ml/tube of wash solution. The beads were then transferred to new tubes and 400μl/tube of colouring solution added (3, 3', 5, 5'-tetramethylbenzidine / sodium acetate buffer, pH 5.5 / 0.015% hydrogen peroxide), followed by incubation for 30 minutes. Development was stopped by the addition of 1ml/tube of 1.3M sulphuric acid, and 100μl aliquots of each specimen transferred to a 96 well plate. The optical densities of each well were determined at 450nm on a microplate reader (Titertek Plus MS2). The levels of MMP 3 in samples of conditioned medium were calculated by comparison to the standard curve, as described above for MMP 1. The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance. The manufacturers of the kit have demonstrated reactivity with active MMP 3 (42%), active MMP 3/TIMP 1 complex (54%) and active MMP 3/TIMP 2 complex (33%), although the measurements obtained with this kit represent the levels of proMMP 3 (as this is used as the standard). The inter-assay coefficient of variance has been demonstrated to be less than 10%.
TIMP 1 levels in samples of conditioned medium collected days 1, 3 and 7 post-seeding into collagen lattices as described in Section 2.10.1.3, were quantitated using a specific ELISA (Amersham). TIMP 1 standards (0 - 50ng/ml) and conditioned medium samples were pipetted into triplicate wells (100µl/well) of a 96 well plate that had been coated with an anti-TIMP 1 antibody and incubated for 2 hours. Conditioned medium samples were assayed at the same dilutions described for MMP 1. The plate was then aspirated and washed four times (400µl/well each wash) with wash buffer (0.01M phosphate buffer pH 7.5 / 0.1% vol/vol Tween 20). An anti-TIMP 1 horseradish peroxidase conjugated antibody was then added to each well (100µl/well) and the plate incubated for 2 hours. Each well was then washed as described above and 100µl of enzyme substrate (3, 3', 5, 5'-tetramethylbenzidine / hydrogen peroxide) added to each well, followed by incubation for 30 minutes. Immunoreactions were then stopped by the addition of 100µl/well of 1M sulphuric acid and the optical densities of each well determined at 450nm. The levels of TIMP 1 in samples of conditioned medium were calculated by comparison to the standard curve, as described above for MMP 1. The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance. The manufacturers of this kit have demonstrated cross-reactivity with free TIMP 1 and TIMP 1 complexed with MMPs 1, 2, 3 and 9, but the kit does not cross-react with TIMP 2. The intra and inter-assay coefficients of variance have been determined as 8.9 - 11.4% and 12.4 - 13.1% respectively, by the manufacturers.
2.10.2 Inhibition of MMP Activity

2.10.2.1 Preparation of MMP Inhibitor and Antibody Solutions

The MMP inhibitors used in this study were Galardin™-MPI, BB-94 and CellTech compound which were generous gifts of Glycomed, California, USA; British Biotechnologies, Oxon, UK and Pfizer, Kent, UK respectively. All of these compounds are hydroxamic acid derived, and are specific, broad spectrum inhibitors of the MMPs (Grobelny et al. 1992; Davies et al. 1993; also see Table 2.3). Stock solutions of Galardin™-MPI (100mM), BB-94 (10mM) and CellTech compound (100mM) were prepared in DMSO. Working solutions of all of these compounds were prepared by diluting stocks in DMEM/10% NCS. The controls for each of these MMP inhibitors consisted of stock solutions of hydroxamic acid (Sigma) dissolved in the same solvent at the same concentrations described above for Galardin™-MPI, BB-94 and CellTech, which were then diluted in DMEM/10% NCS prior to use.

The effects of human recombinant TIMP 1 (a generous gift from Dr. Gill Murphy, Strangeways Laboratories, Cambridge, U.K.) and polyclonal antibodies to MMPs 1, 2, 3 (Biogenesis) upon collagen contraction were also investigated. The antibodies were prepared sodium azide free (courtesy of Dr. Alan Tunnicliffe, Biogenesis), and diluted in NCS prior to use. The control solution for these antibodies consisted of NCS containing 0.14% (wt/vol) purified rabbit IgG (Sigma). This was chosen as a control as the antibodies were rabbit IgGs and the protein content of these antibody solutions was less than 0.14% in each case.
2.10.2.2 MMP Inhibitors and Collagen Contraction

The effects of three different broad spectrum MMP inhibitors (Galardin™-MPI, BB-94 and CellTech) upon collagen contraction were investigated. The inhibition constants of these inhibitors against MMPs are shown in Table 2.3.

Table 2.3: Inhibition constants (Ki's) for synthetic hydroxamic acid derived matrix metalloproteinase inhibitors

<table>
<thead>
<tr>
<th>MMP</th>
<th>Galardin™-MPI</th>
<th>BB-94</th>
<th>CellTech</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP 1 (collagenase)</td>
<td>0.4nM</td>
<td>4nM</td>
<td>150nM</td>
</tr>
<tr>
<td>MMP 2 (72kD gelatinase)</td>
<td>0.5nM</td>
<td>4nM</td>
<td>0.025nM</td>
</tr>
<tr>
<td>MMP 3 (stromelysin)</td>
<td>27nM</td>
<td>20nM</td>
<td>5.9nM</td>
</tr>
<tr>
<td>MMP 9 (92kD gelatinase)</td>
<td>0.2nM</td>
<td>2nM</td>
<td>0.0125nM</td>
</tr>
</tbody>
</table>

To investigate the effects of MMP inhibitors on collagen contraction, triplicate collagen lattices containing 100 000 and 500 000 cells/ml of lattice were prepared as described in Section 2.8.3. The lattices were then overlaid with normal growth medium
containing 100µM hydroxamic acid (controls) or varying concentrations of Galardin™-MPI, BB-94 or CellTech compound (0.1nM - 100µM). Lattice areas were measured days 1, 3 and 7 post seeding as described in Section 2.8.5.

The effects of MMP inhibitors on collagen contraction by cells seeded upon the surface of collagen matrices were also investigated to see if the contraction of matrices by cells with two dimensional contacts with a collagen substrate required MMP activity to affect this contraction. Briefly, collagen lattices were prepared in 48 well plates as described in Section 2.8.3. After the lattices had polymerised 7.5x10⁴ or 1.5x10⁴ cells were seeded onto the surfaces of the lattices and allowed to attach for 30 minutes in medium containing MMP inhibitors at concentrations described above. After this 30 minute incubation, the lattices were carefully released from the sides of the wells using a micropipette tip. Lattice area measurements were made and analysed days 1, 3 and 7 post seeding as described above for cells seeded within collagen lattices.

To investigate if collagen contraction was dependent upon the local extracellular concentration of MMP inhibitors, experiments similar to those above but incorporating hydroxamic acid (final concentration 100µM) or Galardin™-MPI (at final concentrations of 0.1nM -100µM) into the collagen lattices only were also performed. Again the effects of these treatments were monitored by lattice area measurements days 1, 3 and 7 post seeding as described in Section 2.8.5.

2.10.2.3 MMP Inhibitors on MMP Activity Produced During Collagen Contraction.

Samples of conditioned medium were collected from contracting collagen lattices (see Section 2.10.1.3) containing 500 000 cells/ml of lattice at day 7 post seeding.
The samples were then lyophilised, reconstituted in PBS (see Section 2.10.1.3) and subjected to gelatin zymography as described in Section 2.10.1.5. After protein separation and renaturation, the zymogram gels were cut into single lane strips and developed overnight at 37°C in developing buffer alone or developing buffer containing varying concentrations of Galardin™-MPI, BB-94 or CellTech compound (0.1nM - 100μM). MMP activity was then visualised as described in Section 2.10.1.5.

2.10.2.4 TIMP 1, MMP Antibodies and Collagen Contraction.

TIMP 1 was diluted in DMEM/10% NCS prior to its addition to medium overlying lattices (final concentrations of 0.1nM - 1μM). Due to the high molecular weight of MMP antibodies (IgG; M₄ 150 000), the ability of these molecules to penetrate collagen lattices was considered to be potentially low. As a result, the MMP antibodies were combined with the collagen lattice mixture (diluted in NCS/cell suspension) at a range of dilutions (1:320 - 1:20). Triplicate collagen lattices were then seeded with 100 000 cells/ml of lattice into 48 well plates as described in Section 2.8.3, and exposed to normal growth medium. Lattice area measurements were made days 1, 3 and 7 post seeding and analysed as described in Section 2.8.5.

2.10.2.5 Reversibility of MMP Inhibitors

In order to determine if the effects of the MMP inhibitor Galardin™-MPI were long term and reversible, three groups of triplicate collagen lattices (containing 1x10⁵ cells/ml of lattice) were prepared. The first group (controls) were continually exposed to growth medium containing hydroxamic acid (100μM) for 48 days. The second group (long term effects) were continually exposed to Galardin™-MPI (10μM) in growth medium for...
48 days. The third group (reversibility) were initially exposed to Galardin™-MPI (10μM) for 14 days, after which the lattices were washed with PBS (5x3ml) and exposed to growth medium containing 100μM hydroxamic acid, as for controls. Each of the test media overlying lattices was changed every 3 to 4 days. The cells were monitored throughout the culture period by phase contrast microscopy. Changes in collagen lattice area were measured as described in Section 2.8.5, over the 48 day culture period at days 1, 3, 7, 14, 24, 36 and 48 post seeding.

2.10.2.6 Effects of the Addition of MMP Inhibitors to Contracting Collagen Lattices

Briefly, for addition experiments lattices seeded with 1x10^5 cells/ml of lattice were allowed to contract for 1 day prior to the addition of Galardin™-MPI (10μM) or hydroxamic acid (100μM) in DMEM/10% (vol/vol) NCS. The degree of collagen contraction was then followed for a further 44 days. Lattice area measurements were made days 1, 4, 7, 14, 24, 36 and 48 days post seeding as described in Section 2.8.5, and statistically analysed as described in Section 2.9.2.

2.10.3 Specificity of MMP Inhibitors

2.10.3.1 MMP Inhibitors and Cell Viability in Collagen Lattices

The process of collagen contraction has been shown to be dependent upon several factors including cell number, the actin cytoskeleton and the ability of cells to attach to the collagen matrix (see Section 1). Therefore, the effects of MMP inhibitors on these factors was also investigated.
Collagen lattices were seeded with $5 \times 10^5$ cells/ml of lattice into 48 well plates as described in Section 2.8.3, and exposed in triplicate to Galardin™-MPI (0.1nM - 100μM) in DMEM/10% NCS or DMEM/10% NCS containing 100μM hydroxamic acid (control; see Section 2.10.2.1). The viability of cells populating these lattices was then determined 1, 3 and 7 days post seeding using a lactate dehydrogenase (LDH) cytotoxicity kit (Boehringer Mannheim) as described below. Following continual exposure to MMP inhibitor, growth medium overlying lattices was aspirated (and saved for LDH assay) and each lattice washed 5 times with PBS (1ml/wash) prior to the addition of 500μl/well of phenol red and serum free DMEM (Sigma) and incubation for 24 hours. This step was included in the assay as serum has been found to contain high levels of LDH. Cells in lattices exposed to Galardin™-MPI (0.1nM - 100μM) or 100μM hydroxamic acid were then lysed by the addition of 500μl/well of 2% (vol/vol) Triton-X100 in phenol red and serum-free DMEM and incubation at 37°C in 5% CO₂ in air for 30 minutes. The lattices were then checked by phase contrast microscopy to ensure all cells had lysed. Aliquots of medium (100μl) that had been overlying the lattices and lysates from the lattices were then transferred in triplicate to a 96 well plate, i.e., triplicate samples from each of the triplicate lattices. In addition, as a negative control 100μl of phenol red and serum free DMEM containing an equal volume of 2% (vol/vol) Triton X-100 in this medium was transferred to the plate into triplicate wells. To determine the LDH activity in the samples, 100μl/well of reaction mixture (250μl of catalyst diaphrose/NAD⁺ mixture and 11.25ml of dye solution containing iodotetrazolium chloride and sodium lactate) was added. The plate was then incubated for 30 minutes while shielded from light. The optical density of each well was then determined on a microplate reader at 490nm and results expressed as the
mean of triplicate lattices (each read in triplicate) plus or minus the standard error (+/- S.E.). The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.

As cells in this model of collagen contraction do not proliferate (see Section 1), then it was assumed that any differences in cell number due to the cytotoxicity of Galardin™-MPI would be reflected in differences in the levels of LDH that were in either the medium overlying the lattices or recovered upon lysis of the cells populating lattices compared to controls.

2.10.3.2 MMP Inhibitors on Cell Number and Viability in Monolayer Culture

Cells were seeded at 10000 cells/cm² into 24 well plates and allowed to settle overnight. The overlying medium was then aspirated and replaced with either normal growth medium containing 100μM hydroxamic acid (control) or 100μM and 10μM Galardin. Media was changed on the cells every 3 to 4 days. Triplicate wells were harvested by trypsinisation, centrifugation (200g for 8 minutes) and resuspension in PBS containing 0.04% (wt/vol) trypan blue (Sigma) days 1, 7, 14, 36 and 48 days post seeding. Each of the triplicate samples were counted three times on a haemocytometer for total cell numbers and non-viable cells (stained blue). Cell numbers were expressed as the means (+/- standard error) of triplicate counts of triplicate samples (i.e., nine counts in total). Cell viability was expressed as the mean percentage (+/- S.E.) of viable cells of the total cell number from three counts each of the triplicate samples. The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way
analysis of variance (ANOVA). The observed significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.

2.10.3.3 MMP Inhibitors and the Actin Cytoskeleton of Cells in Collagen Lattices and Monolayer Cultures

Collagen lattices containing 1x10^4 cells/ml of lattice that had been exposed to growth medium containing Galardin™-MPI (100μM) or hydroxamic acid (100μM), were harvested days 1, 3 and 7 post seeding. Lattices were then washed in PBS prior to fixation and staining of the actin cytoskeleton as described in Section 2.9.4. For actin cytoskeleton staining in monolayer culture, cells were seeded at 10000 cells/cm^2 in 8 well LabTek chamber slides (Gibco) and allowed to settle overnight. The overlying medium was aspirated and replaced with normal growth medium containing 100μM hydroxamic acid (control) or 100μM Galardin™-MPI. Cells were then harvested day 7 post seeding by the removal of medium, washing with PBS (3 washes of 500μl/well) and fixation in formal saline for 1 hour. The LabTeks were then washed in TBS (3 washes of 500μl/well) prior to staining for filamentous actin using a modification of the method described above. Briefly, cells were stained with 300μl/well FITC-phalloidin (2.5μg/ml) for 30 minutes prior to three washes with TBS (500μl/well). The cells were then incubated in 300μl/well of 7-aminoactinomycin D (100μg/ml) for 30 minutes followed by three washes (500μl/well) and mounting in PBS. Samples were then viewed by confocal microscopy using a MRC 500 (Bio-Rad) confocal visualisation unit mounted over an infinity corrected Axioplan Microscope (Zeiss) fitted with a X63 oil immersion objective, configured as
described previously for FITC visualisation (Entwistle and Noble, 1994) where each image was the average of 128 passes of the laser.

2.10.3.4 MMP Inhibitors and Cellular Attachment to Collagen

A cell adhesion assay (Cytomatrix; Chemicon) was initially employed to investigate the ability of cells to attach to a collagen matrix in the presence of MMP inhibitors. Briefly, collagen type I coated 96 well plates were rehydrated for 15 minutes with 200μl/well of PBS according to the assay manufacturer's instructions. Subconfluent cultures of human Tenon's capsule fibroblasts were harvested by trypsinisation and centrifugation as described in Section 2.1.3, and total cell numbers were determined using a Coulter counter. Cell suspensions (containing 50 000 cells/ml) were prepared in DMEM/10% NCS containing hydroxamic acid/ DMSO (controls; see Section 2.10.2.1), DMEM/10% NCS containing Galardin™-MPI, BB-94 or CellTech (100nM-100μM). One hundred microlitres of each sample was added to triplicate wells, and the cells allowed to settle for 1 and 24 hours at 37°C. After these incubation periods, each well was gently washed with 200μl of PBS (3 washes in total) prior to the addition of 100μl/well of 0.2% (wt/vol) crystal violet in 10% (vol/vol) ethanol and incubation for 5 minutes. The stain was aspirated from the wells, which were then washed three times with 300μl of PBS. The stained cells still attached to the collagen matrix were then lysed by the addition of 100μl/well of 1% (wt/vol) SDS in PBS and incubation for 5 minutes on a shaking platform. The absorbance of the dissolved stain/well was then determined at 540nm using a microplate reader Titertek Multiscan Plus), and expressed as the mean of triplicate wells (+/- S.E.). The data was statistically analysed using computer software (SPSS for Windows; SPSS Inc.) using a one-way analysis of variance (ANOVA). The observed
significance levels from multiple comparisons were adjusted using the Bonferroni test. A probability (p) value of less than 0.05 (p<0.05) was considered as indicating significance.

The ability of cells exposed to MMP inhibitors to attach to their surrounding collagen matrix was further investigated by TEM. Collagen lattices containing 1x10⁵ cells/lattice were prepared as described in Section 2.8.3, and exposed to normal growth medium containing 100μM hydroxamic acid or 100μM Galardin™-MPI. Lattices were harvested and processed days 1, 3 and 7 post seeding for TEM as described in Section 2.9.7. The number of collagen fibres associated with the cell surface in control lattices and those exposed to Galardin™-MPI was assessed from the electron micrographs produced.

2.10.4 MMP Inhibitors and Collagen Contraction, Morphology, Movement and Growth Arrest

2.10.4.1 MMP Inhibitors and Collagen Contraction by Fibroblasts Derived from Different Tissue Sites and Species

Collagen lattices were seeded with 5x10⁵ cells/ml of lattice, derived from different tissue sites and species including human corneal and dermal fibroblasts, rat dermal, endotendon and synovial sheath fibroblasts and rabbit conjunctival fibroblasts, as described in Section 2.8.3. Human corneal and rabbit conjunctival fibroblasts were propagated from tissue explants as described in Section 2.1.1, whereas human dermal, rat dermal, endotendon and synovial sheath fibroblasts were kind gifts from Dr. Umraz Khan and Mrs. Avril Burt (Phoenix Tissue Repair Unit, University College, London). The lattices were exposed to DMEM/10% NCS containing 100μM hydroxamic acid (controls)
or 100μm Galardin™-MPI. Lattice areas were measured days 1, 3 and 7 post seeding as described in Section 2.8.5, and analysed as described in Section 2.9.2.

2.10.4.2 MMP Inhibitors and Cell Morphology / Movement Within Collagen Lattices

Collagen lattices seeded with 100 000 cells/lattice were prepared as described in Section 2.8.3. Lattices were either overlaid with normal growth medium (controls) or growth medium containing Galardin™-MPI (1-100μM), BB-94 (1-100μM), CellTech (1-100μM), 100μM hydroxamic acid (controls; see Section 2.10.2.1), TIMP 1 (0.1nM - 1μM), MMP 1 antibody, MMP 2 antibody, MMP 3 antibody (1:320 - 1:20) or 0.14% purified rabbit IgG (diluted 1:20; antibody control, see Section 2.10.2.1). The morphology of cells populating all of these lattices was monitored at 2, 4, 24, 48 and 168 hours post seeding by phase contrast microscopy (see Section 2.9.6).

The movement of cells within the lattices exposed to growth medium containing 100μM hydroxamic acid (control) or 100μM Galardin™-MPI was monitored for 40 hours by time lapse video. Briefly, collagen lattices floating in a minimal volume of growth medium were placed into a humidified chamber maintained at 37°C and 5% CO₂ in air, mounted on a phase contrast microscope (Zeiss Axiovert; Zeiss), attached to a video camera. Cells within the matrix were then video taped for 40 hours.

2.10.4.3 MMP Inhibitors and Cell Morphology / Movement in Monolayer Culture

Cells were seeded at a density of 10 000 cells/cm² into 24 well plates and allowed to settle overnight. The overlying medium was then aspirated and replaced with
either normal growth medium containing 100\mu M hydroxamic acid (MMP inhibitor control; see Section 2.10.2.1), a solution of 0.14\% (wt/vol) rabbit IgG diluted 1:20 in NCS (MMP antibody control; see Section 2.10.2.1), 100\mu M Galardin™-MPI, 100\mu M BB-94, 100\mu M CellTech compound, or rabbit polyclonal antibodies to MMPs 1, 2, 3 (all diluted 1:20 in collagen lattice mixture). The plates were then incubated for 7 days at 37°C in 5\% CO₂ in air, prior to documentation of cellular morphology by phase contrast micrography (see Section 2.9.6) and cell number and cell viability determinations as described in Section 2.9.3. The media were changed on each well at day 3 post seeding.

The movement of cells in monolayer culture exposed to growth medium containing 100\mu M hydroxamic acid (control) or 100\mu M Galardin™-MPI was monitored for 40 hours by time lapse video. Briefly, cells were seeded into 6 well plates (10 000/well) in growth medium containing hydroxamic acid or Galardin™-MPI and allowed to attach for 30 minutes. The plate was then placed into a humidified chamber maintained at 37°C and 5\% CO₂ in air, mounted on a phase contrast microscope (Zeiss Axiovert; Zeiss), attached to a video camera. Cells within the wells were then video taped for 40 hours.

2.10.4.4 Exposure to Antimetabolites and MMP / TIMP 1 Protein Production

The effects of exposure to 5FU (0.25 - 25mg/ml) and MMC (0.001 - 0.1mg/ml) on MMP and TIMP 1 protein production in samples of conditioned medium during collagen contraction, were initially investigated using specific ELISAs as described in Sections 2.10.1.3 and 2.10.1.6. Analysis of the MMP profile and biological activity of these samples of conditioned medium was further investigated by gelatin zymography as described in Section 2.10.1.5.
3. RESULTS

3.1 Effects of Antimetabolites on Molecular Aspects of Fibroblast Wound Healing Behaviour

3.1.1 Total RNA and Protein Production

Throughout the 48 day period of RNA and conditioned medium collection, all cells appeared viable morphologically. This was confirmed using a trypan blue dye exclusion test, which showed that there was no significant (p>0.05) difference in the percentage viability of cells between different treatments. Exposure to 5FU resulted in an initial significant (p<0.05) depression of total RNA production compared to controls (Figure 3.1). This was then followed by a significantly marked increase (p<0.05) and eventual decrease to control levels by day 48 post-exposure (for 0.25 and 2.5mg/ml 5FU only). Exposure to 25mg/ml 5FU elicited a biphasic effect on total RNA production, with peak expression days 14 (p>0.05) and 48 (p<0.05). Following an initial increase (p<0.05) in total protein production by cells exposed to 5FU compared to controls, cellular protein production (Figure 3.1) was similar to that of total RNA. In each case an increased production of total RNA preceded a resultant increase in total protein production. Exposure to MMC resulted in similar effects to those of 5FU, on total RNA and total protein (Figure 3.1) production. It therefore appeared that exposure to antimetabolites elicited a significant increase in total RNA production peaking around 14 days post-exposure, which then resulted in a subsequent increase in total protein production around day 24 post-exposure. Notably, exposure to the highest concentrations of 5FU or MMC, unlike lower concentrations, while reducing total RNA levels to the greatest extent resulted in the highest protein production levels.
Figure 3.1: Effects of antimetabolites on long term RNA and protein production by human tenon's capsule fibroblasts

Graphs show that exposure to antimetabolites resulted in an increase in RNA production around day 14 post exposure, followed by a decrease in production towards control levels. These alterations in RNA production were reflected in subsequent long term protein production.
3.1.2 Growth Factor Production

3.1.2.1 Transforming Growth Factor β1 (TGFβ1)

Exposure to high concentrations of 5FU (25mg/ml) significantly reduced (p<0.05) TGFβ1 mRNA levels compared to controls up to day 24 (Figure 3.2). Exposure to lower concentrations of 5FU (2.5 and 0.25mg/ml) while initially reducing TGFβ1 mRNA levels compared to controls (p<0.05), showed a marked increase by day 14 post-exposure, followed by a reduction towards control levels up to day 48. MMC significantly reduced (p<0.05) TGFβ1 mRNA levels up to day 24, compared to controls, at a concentration of 0.1mg/ml (Figure 3.2). MMC (0.01 and 0.001mg/ml) although initially reducing TGFβ1 mRNA levels, resulted in a significant increase (p<0.05) in these levels at day 14, followed by a decrease towards control levels.

Only exposure to 0.25mg/ml 5FU did not initially depress TGFβ1 protein levels compared to controls (p>0.05; Figure 3.2). Unlike control cultures, cells exposed to 5FU then significantly increased (p<0.05) their TGFβ1 production, followed by a decrease towards control levels for 0.25 and 2.5mg/ml 5FU. TGFβ1 production by cells exposed to 25mg/ml 5FU appeared to show a biphasic response, peaking initially at day 24 post exposure and then at day 48. The profile of TGFβ1 production by cells exposed to 5FU was very similar to that observed for total protein production in Section 3.11, and shown in Figure 3.1. Exposure to MMC, like 5FU, resulted in an initial increase (p<0.05) in TGFβ1 protein production compared to controls (Figure 3.2). TGFβ1 levels peaked maximally at day 7 post MMC exposure for all concentrations tested, followed by a sustained decrease days 24, 36 and 48 for 0.001, 0.01 and 0.1mg/ml MMC respectively.
After these times, exposure to both 0.1 and 0.01mg/ml MMC resulted in a further increase (p<0.05) in TGFβ1 levels up to 48 days.

3.1.2.2 Basic Fibroblast Growth Factor (bFGF)

bFGF protein production by cells exposed to 5FU was initially significantly elevated (p<0.05) compared to PBS controls regardless of the concentration used (Figure 3.3). Unlike the highest concentration of 5FU, bFGF protein levels showed a sustained decrease to that of controls by day 48 post-exposure to 0.25 and 2.5mg/ml 5FU. Exposure to 25mg/ml 5FU resulted in a significantly increased bFGF production (p<0.05) peaking maximally at day 36, followed by a decrease in levels day 48 post exposure. Exposure to MMC resulted in an initial significant (p<0.05) increase in bFGF production (up to 10 fold) compared to controls, that appeared to be MMC concentration dependent (Figure 3.3). Following this initial increase, bFGF production by cells exposed to all concentrations of MMC showed a continual decrease to control levels (p<0.05) by days 14, 24 and 36 for 0.001, 0.01 and 0.1 mg/ml MMC respectively.

It was not possible to measure bFGF mRNA levels by QCRT-PCR during this study, as Dr. Roy Tarnuzzer (University of Florida, U.S.A.) was unable to make this template available due to technical problems. Therefore only the protein results are presented.
Figure 3.2: Effects of antimetabolites on long term TGFβ1 mRNA and protein production by human tenon's capsule fibroblasts

Graphs show exposure to antimetabolites affected both TGFβ1 mRNA and protein levels. Although high concentrations of 5FU (25mg/ml) reduced TGFβ1 mRNA to very low levels throughout the 48 day assay period, it resulted in a sustained increase of TGFβ1 protein production over the same period. Lower concentrations of 5FU resulted in an initial decrease in TGFβ1 mRNA levels, a subsequent peak at day 14 and then decreased towards control levels. A similar profile was observed with TGFβ1 protein levels. Exposure to 0.1mg/ml MMC reduced TGFβ1 mRNA levels, TGFβ1 protein being initially raised but subsequently decreasing towards control levels. Exposure to lower concentrations of MMC (0.01 and 0.001mg/ml) elicited similar effects on TGFβ1 mRNA and protein profiles, to low concentrations of 5FU.
Graphs show that exposure to antimetabolites resulted in an initial increase in bFGF production compared to controls, regardless of the agent used or its concentration. Exposure to high (25mg/ml) concentrations of 5FU resulted in a prolonged increase in bFGF protein production, peaking maximally at day 36 post exposure. bFGF production following exposure to lower concentrations of 5FU, and all concentrations of MMC, showed a sustained decrease towards control levels following this initial rise.
3.1.2.3 Epidermal Growth Factor (EGF)

The levels of EGF mRNA and protein produced by cells exposed to either 5FU, MMC or PBS (controls) were not detectable, by the QCRT-PCR or ELISA techniques used, up to 48 days post-exposure to these agents.

3.1.3 Growth Factor Receptor Expression

3.1.3.1 Transforming Growth Factor β (TGFβ) Type II Receptor

Exposure to 25 and 2.5mg/ml 5FU caused an initial significant reduction (p<0.05) in TGFβ type II receptor mRNA levels (Figure 3.4), while exposure to 0.25mg/ml caused an initial significant increase (p<0.05). Following these initial effects the levels of TGFβ type II receptor mRNA decreased further to day 24, mRNA levels then beginning to steadily increase over the remainder of the 48 day period although still reduced compared to controls. At the protein level, exposure to 5FU caused a significant reduction (p<0.05) in TGFβ type II receptor expression. The levels of this receptor subsequently continued to increase towards control levels over the 48 day period, with only cells exposed to 0.25mg/ml 5FU reaching control levels. Exposure to MMC did not appear to have as marked an effect on TGFβ type II receptor mRNA levels (Figure 3.4) as 5FU. At the protein level, receptor numbers were initially significantly decreased (p<0.05) with cells recovering expression to control levels (in the case of 0.001 and 0.01mg/ml MMC) or in excess of control levels (for 0.1mg/ml MMC).
3.1.3.2 Fibroblast Growth Factor (FGF) Receptor

The expression of FGF receptor protein following exposure to antimetabolites (Figure 3.5) was similar to that seen for the TGFβ type II receptor. Exposure to 5FU initially significantly reduced (p<0.05) the number of FGF receptors, followed by a recovery to control levels by day 48. Following exposure to MMC, FGF receptor numbers although initially reduced (p<0.05) reached levels in excess of controls by day 48 (for 0.1 and 0.01mg/ml MMC only).

It was not possible to measure FGF receptor mRNA levels by QCRT-PCR during this study, as Dr. Roy Tamuzzer (University of Florida, U.S.A.) was unable to make this template available due to technical problems. Therefore only the protein results are presented.

3.1.3.3 Epidermal Growth Factor (EGF) Receptor

Exposure to 5FU appeared to reduce both EGF receptor mRNA and subsequent EGF receptor protein expression in a concentration dependent manner (Figure 3.6). Whereas cells exposed to low concentrations of 5FU (0.25 and 2.5mg/ml) appeared to recover expression, the number of receptors expressed by those exposed to high 5FU (25mg/ml) were significantly reduced (p<0.05) throughout the 48 day period. Exposure to MMC resulted in an initial reduction in receptor numbers (Figure 3.6), with cells recovering this expression by day 24. It was noted after day 24 that although cells exposed to MMC either had similar or significantly reduced (p<0.05) levels of EGF receptor mRNA expression, the actual number of receptors expressed was significantly elevated (p<0.05) compared to controls.
Graphs show that exposure to antimetabolites altered TGFβ type II receptor mRNA and protein levels. Exposure to 5FU reduced TGFβ type II receptor mRNA and protein levels, with cells appearing to recover production towards control levels by day 48. Although MMC initially reduced TGFβ type II receptor protein levels, this was not reflected in mRNA production. By day 48, cells exposed to MMC appeared to have recovered receptor mRNA expression to similar levels to those of controls. It was noted that exposure to MMC resulted in a significantly increased (p<0.05) level of expression of TGFβ receptor protein days 24 to 48.
Figure 3.5: Effects of antimetabolites on long term bFGF receptor protein expression by human tenon's capsule fibroblasts

Graphs show that exposure to antimetabolites initially reduced bFGF receptor protein levels in a concentration dependent manner. The expression of these receptors by cells exposed to 5FU recovered to control levels by day 48. Cells exposed to MMC exhibited receptor expression in excess of controls by day 24, these levels continuing to increase for 0.1 and 0.01mg/ml but decreasing for 0.001mg/ml to control levels by day 48.
Figure 3.6: Effects of antimetabolites on long term EGF receptor mRNA and protein expression by human tenon's capsule fibroblasts

Graphs show exposure to antimetabolites affected long term EGF receptor mRNA and protein expression. Exposure to 5FU or MMC appeared to reduce both EGF receptor mRNA and subsequent protein expression in a concentration dependent manner. Exposure to high concentrations of MMC significantly reduced (p<0.05) mRNA levels throughout the 48 day period, while receptor protein expression was observed to be in excess of control levels days 24 to 48.
3.1.4 Extracellular Matrix Molecule Production

An ELISA technique, developed by Dr. Peter Constable at the Institute of Ophthalmology, was used to quantitate the production of collagen type I and collagen type III by cells exposed to antimetabolites or PBS (controls). This optimal conditions for this technique were pre-determined by Dr. Constable, as illustrated in Figure 3.7. As stated in the materials and methods section of this thesis, the optimal dilutions of the primary and secondary antibodies were found to be 1:2000 and 1:8000 respectively. Additionally, the optimal development period was found to be 30 minutes. Although the primary antibodies used in this study exhibited cross-reactivity with collagen type I or III antigens, no cross-reactivity with fibronectin was observed. The levels of production of collagen type I and type III proteins in samples of conditioned medium were determined by subtraction of the value of cross-reactivity obtained with the respective antigens. It should also be noted that the values obtained for collagen type I or III in conditioned media were compared at the same dilution, preferably on parts of the standard curve were cross-reactivity of the primary antibodies was least.
3.1.4.1 Collagen Type I

Control cultures (PBS) produced increasing levels of collagen type I mRNA throughout the 48 day culture period, the production of collagen type I protein following a similar profile (Figure 3.8). Exposure to high concentrations of 5FU (25mg/ml) although significantly elevating (p<0.05) collagen type I mRNA levels at day 3, caused a sustained significant decrease (p<0.05) in mRNA levels over the remainder of the 48 day assay period. Analysis of collagen type I protein production indicated a similar profile to mRNA, with exposure to 25mg/ml 5FU significantly reducing (p<0.05) protein production compared to controls. Exposure to lower concentrations of 5FU (2.5 and 0.25mg/ml) significantly decreased (p<0.05) the levels of both collagen type I mRNA and protein throughout the 48 day assay period.

Similar results were seen upon exposure to MMC (Figure 3.8), resulting in a significant reduction (p<0.05) in both collagen type I mRNA and protein levels over the 48 day assay period.
Figure 3.7: Determination of optimal conditions for collagen type I and collagen type III ELISAs

Graphs show optimal dilutions for primary and secondary antibodies used for experimentation were 1:2000 and 1:8000 respectively. The optimal development period was 30 minutes. The anti-collagen type III primary antibody cross-reacted with collagen type I antigen, as did the collagen type I antibody with collagen type III antigen. The actual degree of cross-reactivity that occurred during each assay was taken into account when calculating subsequent data. (Data for graphs kindly provided by Dr. P. Constable).
Figure 3.8: Effects of antimetabolites on long term collagen type I mRNA and protein production by human tenon's capsule fibroblasts

Graphs show that exposure to both 5FU and MMC significantly reduced (p<0.05) the production of collagen type I mRNA and protein, compared to controls, up to 48 days post-treatment.
3.1.4.2 Collagen Type III

The maximal production of both collagen type III mRNA and protein occurred at day 3, their production continually decreasing over the remainder of the 48 day assay period (Figure 3.9). Exposure to high concentrations of 5FU (25mg/ml) caused an initial significant decrease (p<0.05) in collagen type III mRNA levels, followed by a sustained increase in mRNA production days 24-48. At the protein level, collagen type III production was seen to be initially high (day 3), then reduced (day 7), followed by a significant increase (p<0.05) in production at day 14, and finally a reduction towards control levels over the remainder of the assay. Exposure to lower concentrations of 5FU (2.5 and 0.25mg/ml) resulted in a reduction of collagen type III mRNA levels, while protein levels peaked at day 7 followed by a decrease towards control levels over the remainder of the 48 day assay period.

Exposure to MMC reduced collagen type III mRNA levels, only cells exposed to 0.1mg/ml MMC appearing to recover production towards control levels (Figure 3.9). Collagen type III protein production by cells exposed to MMC peaked maximally at day 7 followed by a reduction towards control levels, at all concentrations of the antimetabolite tested.
Figure 3.9: Effects of antimetabolites on long term collagen type III mRNA and protein production by human tenon's capsule fibroblasts

Graphs show exposure to antimetabolites affected both collagen type III mRNA and protein levels. High concentrations of 5FU (25mg/ml) resulted in an increase in both mRNA and protein levels, while lower concentrations although decreasing mRNA levels resulted in a peak protein production at day 7, followed by a sustained decrease to control levels. Following exposure to MMC, at all concentrations tested, a similar profile of collagen type III mRNA and protein production to that elicited by lower concentrations of 5FU (2.5 and 0.25mg/ml) was seen.
3.1.4.3 Fibronectin

Control cultures produced increasing levels of fibronectin mRNA up to day 14, followed by a sustained decrease up to day 48 (Figure 3.10). Exposure to 5FU, at all concentrations tested, significantly reduced (p<0.05) fibronectin mRNA production up to day 36 post-exposure, production then exceeding that of controls (for 25 and 0.25mg/ml 5FU only). The levels of fibronectin protein production were also significantly reduced (p<0.05) upon exposure to 5FU, with production beginning to increase by day 48. Similar results to those obtained with 5FU, were observed following exposure to MMC (Figure 3.10).
Graphs show that both 5FU and MMC significantly reduced (p<0.05) fibronectin mRNA and protein production over the 48 day assay period.
3.2 Effects of Antimetabolites on Cellular Aspects of Fibroblast Wound Healing

Behaviour

3.2.1 Proliferation

Differences in the morphologies of cells exposed to 5FU and MMC were noted over the 48 day culture period, compared to controls. Cells exposed to PBS (controls) assumed a characteristic fibroblast 'spindle shaped' morphology and were densely packed. Cells exposed to high concentrations of 5FU (25mg/ml) or MMC (0.1mg/ml) appeared abnormally large and spread, some cells appearing multinucleated. The morphology of cells exposed to lower concentrations of these agents were more similar to those assumed by controls.

Exposure to 5FU resulted in the significant (p<0.05) growth arrest of cultures for 7, 14 and 48 days for 2.5, 0.25 and 25mg/ml 5FU respectively (Figure 3.11). Only cells exposed to 2.5mg/ml 5FU reached the levels of control cell numbers by 48 days post-exposure. For cells exposed to MMC, only 0.1mg/ml appeared to result in cellular growth arrest (up to 36 days), these cells then beginning to proliferate (Figure 3.11). Although exposure to 0.001 and 0.01mg/ml MMC did not appear to result in appreciable growth arrest, the levels of proliferation were significantly reduced (p<0.05) compared to controls for 24 and 48 days respectively.
Figure 3.11: Effects of antimetabolites on long term human tenon’s capsule fibroblast proliferation

Graphs show that single five minute exposures to 5FU or MMC had long term effects on fibroblast proliferation. Low concentrations of 5FU (0.25 and 2.5mg/ml) appeared to growth arrest cells for 7 - 14 days, followed by recovery of proliferation. Although cells exposed to 2.5mg/ml 5FU fully recovered, those exposed to 0.25mg/ml 5FU did not compared to controls by day 48. Cells exposed to high concentrations of 5FU (25mg/ml) appeared to be growth arrested for 48 days.

Long term inhibition of proliferation was achieved with MMC, cells exposed to 0.001mg/ml fully recovering proliferation relative to controls by day 36. Exposure to 0.01mg/ml MMC still significantly (p<0.05) inhibited proliferation by day 48, although some recovery of proliferation was seen. Cells exposed to 0.1mg/ml MMC appeared growth arrested up to day 36 followed by a partial recovery of proliferation, although the degree of proliferation was still significantly (p<0.05) reduced by day 48.
3.2.2 Fibroblast Settlement and Migration

Using a chequerboard analysis for migration it was seen that an increasing number of cells migrated across the membrane towards the higher concentrations of foetal calf serum (FCS), indicating a predominantly chemotactic response (Table 3.1). As the concentrations of FCS in each well (top and bottom) were increased in equal amounts a small increase in the number of cells migrated occurred, indicating a small chemokinetic response (Table 3.1). It was therefore concluded from this data that human Tenon's capsule fibroblast migration to FCS was primarily via chemotaxis. In addition to this, the maximal migration of cells from serum-free medium towards FCS occurred at 20% (vol/vol) FCS. As a result, a concentration of 20% (vol/vol) FCS in the bottom well of the migration chamber was used for all subsequent experiments. The optimal length of the migration assay was determined by harvesting membranes at various time points (4, 8 and 16 hours post seeding), 16 hours subsequently being chosen for experimentation.

Exposure to 5FU or MMC did not appear to affect the morphology of settled or migrated cells compared to controls by light microscopy. Additionally, using scanning electron microscopy the majority of cells settled onto the membrane appeared elongated and stellate. Several 'shorter' cells were observed that exhibited numerous surface processes and appeared to be migrating through pores in the membrane (Figure 3.12).

Exposure to 5FU or MMC, at all concentrations tested, did not significantly affect (p>0.05) the settlement of cells during the migration assay compared to controls. The migration of cells exposed to 5FU was significantly reduced (p<0.05) up to 48 days compared to PBS controls, for all concentrations of 5FU tested (Figure 3.12). However, cells exposed to 5FU did appear to partially recover their ability to migrate towards FCS
(80% for 0.25 and 2.5mg/ml and 40% for 25mg/ml) over the 48 day period. The inhibitory effects of MMC on migration were less marked than those of 5FU (Figure 3.12), although still significantly reduced (p<0.05) compared to controls at day 48.

From both proliferation and the above migration data, it appears that at high concentrations of antimetabolites only partial recovery of proliferative and migratory capacity occurs. Interestingly, at lower concentrations the 'lag-phase' in the recovery of these cellular functions appear to be very similar in duration.

3.2.3 Extracellular Matrix Contraction

During the wound healing process, fibroblasts are stimulated to perform a variety of functions including increased proliferation. The transition of fibroblasts from a 'resting' or quiescent state (G0 in the cell cycle) into an initially 'active' state (G1 in the cell cycle) is characterised by an increase in metabolic activity (see Section 1). Also, this increase in metabolic activity (cellular activation) must occur in order for these cells to carry out other healing functions such as migration, ECM contraction and the production of ECM. Additionally, it has been suggested that a more aggressive healing response may occur in vivo as a result of increased preoperative cellular activation and subsequent post-operative scarring (Khaw et al. 1994a). Therefore this part of the study investigated not only the effects of antimetabolites on ECM contraction, but also if these effects were dependent upon the initial state of cellular activation.
Table 3.1: Chequerboard analysis of human tenon's capsule fibroblast migration to foetal calf serum (FCS).

<table>
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<td>21 (+/-4)</td>
<td>38 (+/-7)</td>
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<td>191 (+/-18)</td>
<td>132 (+/-4)</td>
<td>94 (+/-12)</td>
<td>45 (+/-7)</td>
</tr>
</tbody>
</table>

The upper chambers were filled with cells in media containing varying amounts of FCS. Lower chambers were filled with media containing the appropriate concentration of FCS.

Migration to FCS consisted of a small chemokinetic response, characterised by an increase in migrated cell numbers as the concentration of FCS was increased by the same amount in the upper and lower chambers (shaded boxes). The migration of cells to FCS was predominantly a chemotactic response as the number of cells migrating across the membrane significantly (p<0.05) increased with increasing concentrations of FCS. Migration appeared to peak maximally at 20% FCS, falling at higher concentrations (50% FCS).

These results suggest that the migration of Tenon's capsule fibroblasts to FCS is predominantly a chemotactic response, stimulated maximally at a concentration of 20% FCS.
Cells migrating through pores had numerous surface processes and appeared 'shorter' in contrast to non-migrating cells, which were elongated / stellate in morphology. Scale bar = 10µm.

Graphs show that single five minute exposures to antimetabolites had long term effects on fibroblast migration. Cells exposed to 5FU partially recovered migratory ability, although they were still significantly (p<0.05) inhibited at day 48 at all concentrations tested, compared to controls. Exposure to MMC had less marked effects, although cell migration was still significantly (p<0.05) inhibited at day 48. Exposure to 5FU or MMC did not affect the settlement of cells, or their morphology, compared to controls.
3.3.1 Characterisation of Activated and Nonactivated Fibroblasts

Prior to seeding into collagen lattices, the biological differences between monolayers of activated and nonactivated fibroblasts were partially characterised. All activated cells expressed Ki67, while significantly fewer (p<0.05) nonactivated cells expressed Ki67 (Figure 3.13). Activated cells may therefore be considered to be proliferating, that is, in G1, G2, S, or M phases of the cell cycle. Nonactivated cells, as a population, may be considered non-proliferating or quiescent cells (in the G0 phase of the cell cycle). Collagen lattices seeded with these two different cell preparations will subsequently be referred to as activated and nonactivated collagen lattices in the following sections. Additionally, using scanning electron microscopy numerous cell surface processes were noted on activated but not activated cells. These findings are similar to those of Alexander et al (1989) who noted that activated trabecular meshwork cells exhibited numerous surface processes by scanning electron microscopy.

3.2.3.2 Collagen Lattice Contraction

In the collagen contraction model used in these experiments with cells seeded within lattices, it was found that contraction as has been described by other researchers (see Section 1), was dependent on cell number (Figure 3.14). A seeding density of 5x10^5 cells/ml of lattice was chosen for this series of experiments, as the degree and rate of collagen contraction in these lattices would allow the easy detection of inhibitory effects. During the process of collagen contraction in control lattices, the cells appeared to undergo a series of morphological changes. Following an initial rounded morphology immediately post seeding the cells began to spread, producing cytoplasmic
processes within 2 hours. The cells then spread further appearing stellate within 24 hours, followed by further elongation and adoption of a stellate/bipolar morphology 24 hours onwards (also see Figure 3.31).

Upon exposure to 5FU, MMC or water (control), all lattices showed a decrease in area 1 day after treatment. A difference in the contractile capacity of nonactivated and activated cells in control lattices was noted (see Figures 3.15 and 3.16). The differences in contractile ability between these cell preparations was found not to be the result of decreased cell viability, as determined by trypan blue staining, both before lattice seeding and after treatment. Collagen lattices also showed no decrease in mean area immediately after five minute exposures to 5FU, MMC, or water (controls). In addition, lattices containing no cells and those containing no cells and exposed to the highest concentrations of 5FU or MMC for five minutes did not show any significant (p>0.05) change in mean area during the 7 day culture period.
Figure 3.13: Characterisation of nonactivated and activated monolayer cultures

Expression of Ki67 in nonactivated human tenon's capsule fibroblasts

Positive nuclei gave a brown reaction product (arrows) while negative nuclei gave no reaction product (arrowheads)

Expression of Ki67 in activated human tenon's capsule fibroblasts

Both photographs were taken at the same original magnification. Scale bar = 25µm.

Both activated and nonactivated cells were viable and morphologically similar prior to seeding into collagen lattices. The number of activated cells staining for Ki67 was significantly (p<0.05) higher than in their nonactivated counterparts. Additionally, the intensity of staining (within the same staining run) was greater in activated cells.
Figure 3.14: *In vitro* model of collagen contraction

**Contraction of collagen lattices**

Collagen lattices seeded with $5 \times 10^5$ cells/ml of lattice showed a marked reduction in lattice area over the 7 day assay period. Scale bar = 10mm.

**Effects of seeding cell density on collagen lattice contraction**

Graph shows that increasing lattice cell number resulted in a significantly ($p<0.05$) increased degree of collagen contraction over the 7 day assay period.
3.2.3.3 Effects of 5FU on Activated and Nonactivated Collagen Lattice

Contraction, Cell Viability, and Number

Single five minute exposures to 5FU caused a significant inhibition of contraction, at all concentrations tested, in both activated (p<0.05) and nonactivated (p<0.05) collagen lattices 7 days after treatment, compared to controls (Figure 3.15). The degree of contraction was greater in collagen lattices containing activated cells than in their nonactivated counterparts at all concentrations of 5FU tested.

The cellular viability and cellularity of activated lattices were not adversely affected by exposure to 5FU (p>0.05). However, the cellular viability of nonactivated lattices was significantly decreased (p<0.05) by exposure to 25 and 1mg/ml of 5FU, these cells appearing rounded by phase contrast microscopy. Cellularity in these lattices was only significantly (p<0.05) affected with high (25mg/ml) concentrations of 5FU.

It therefore appears in the model used that on a cell per cell basis, activated cells are able to contract a collagen matrix to a greater extent than nonactivated cells over the 7 day assay period. The inhibitory effects of 5FU on contraction appear to occur much earlier in this process for nonactivated cells than their activated counterparts. Additionally, exposure to 5FU inhibited contraction of collagen by activated cells by a mechanism(s) other than direct cytotoxicity, whereas this appeared only to be true for nonactivated cells at concentrations of 5FU below 1mg/ml.
Graphs show that single five minute exposures to 5FU significantly (p<0.05) inhibited contraction compared to controls. Inhibition of contraction appeared to occur earlier in lattices populated with nonactivated cells. 5FU only affected cell viability and cellularity in lattices seeded with nonactivated cells. 5FU appeared to inhibit collagen contraction in lattices containing nonactivated cells by causing cell death, down to concentrations of 1mg/ml. This suggests that 5FU inhibited contraction in all of the other activated and nonactivated lattices via antimetabolic effects. The anti-contractile effects of 5FU were not due to inhibition of proliferation within the lattices, as cells do not proliferate in the model used in this study.
3.2.3.4 Effects of MMC on Activated and Non activated Collagen Lattice

Contraction, Cell Viability, and Number

A significant inhibition of contraction ($p<0.05$) occurred in both activated and nonactivated (Figure 3.16) collagen lattices compared to controls upon five minute exposures to MMC. The degree of contraction at all concentrations of MMC was, as for 5FU, greater in lattices seeded with activated cells than in those seeded with nonactivated cells. Exposure to MMC was significantly cytotoxic ($p<0.05$) in lattices seeded with activated (Figure 3.16) and nonactivated cells. Exposure to MMC also resulted in a significant decrease ($p<0.05$) in cellularity in all lattices. As for 5FU, the anti-contractile effects of MMC appeared to be more pronounced in lattices seeded with nonactivated cells compared to their activated counterparts. However, the primary mechanism of inhibition of contraction appeared to be the cytotoxic effects of MMC, which again were more pronounced in lattices seeded with nonactivated cells.
Figure 3.16: Effects of the antimetabolite MMC on activated and nonactivated fibroblast mediated collagen contraction, lattice cell viability and cellularity

Graphs show that MMC inhibits contraction by activated and nonactivated cells, primarily via cytotoxic effects. The inhibition of contraction appeared to occur earlier for lattices seeded with nonactivated cells. As for 5FU (Figure 3.15), lattices seeded with activated cells appeared to carry out some contraction of the lattices before the inhibitory effects of MMC became apparent.
3.2.3.5 Effects of 5FU and MMC on Cellular Morphology

Collagen lattices that had been exposed to 5FU, MMC and water were harvested 7 days after treatment for SEM. The morphological characteristics of cells populating the surfaces of activated lattices treated with 5FU were similar to those populating nonactivated lattices, as were the characteristics of those exposed to MMC. The cells populating the surfaces of lattices exposed to 25mg/ml of 5FU exhibited a range of morphologies including rounded, monopolar, flattened, bipolar, and stellate cells, some of which exhibited long cytoplasmic processes (Figure 3.17). The morphology of cells exposed to a five minute treatment with 0.01 mg/ml of 5FU closely resembled that of controls. Cells on the surfaces of lattices treated with MMC exhibited a variety of morphologies including rounded, flattened - rolled up, monopolar, and bipolar cells (Figure 3.18). The cells on control lattices exhibited a characteristic fibroblast 'streaming' appearance and had a predominantly bipolar elongated morphology with some cells exhibiting overlapping processes (Figure 3.17).

The density of cells on the surfaces of lattices exposed to water appeared to increase over the 7 day assay period, which was associated with a decrease in lattice area. The density of cells on lattices exposed to high concentrations of 5FU and MMC were decreased by day 7 compared to controls. For 5FU samples this appeared to be due to differences in lattice area, as the cells appeared morphologically viable and there was no decrease in cell viability or number over the 7 day assay period (see Figure 3.15). For high concentrations of MMC however, the decrease in cell surface density appeared to be mainly due to cytotoxic effects. These SEM observations were corroborated by phase contrast microscopy observations.
Cells were viewed within collagen lattices by phase contrast microscopy up to 7 days following exposure to 5FU, MMC or water. Cells populating lattices regardless of treatment or of the degree of cellular activation, exhibited cytoplasmic extensions into the surrounding collagen matrix within 6 hours. Cells in control lattices exhibited primarily a stellate morphology 24 hours after treatment, the majority of cells assuming a bipolar morphology within 48 hours of treatment. It was noted particularly in lattices treated with MMC, that cells located at the lattice-medium interface assumed a spherical morphology after 24 hours (suggesting cell death), compared to a heterogeneous appearance of stellate and spherical cells located mid-lattice. However, cells located mid-lattice did subsequently assume a similar spherical structure approximately 48 hours after treatment.
Figure 3.17: Effects of 5FU on the morphology of activated cells populating collagen lattices by scanning electron microscopy

Cells exposed to 25 mg/ml 5FU

Cells exposed to 0.01 mg/ml 5FU

Cells on the surface of these lattices appeared stellate. There appeared to be a reduced lattice surface cell density compared to controls.

Cells had an elongated bipolar morphology similar to that of controls. The lattice surface cell density was more similar to that of controls than that of 25 mg/ml 5FU.

Cells exposed to water or PBS (control)

Cells appeared elongated by day 7 post seeding. The surface of the lattice was densely populated with cells. The differences seen in surface cell density appeared to be related to lattice area and not lattice cellularity (also see Figure 3.15).
Figure 3.18 : Effects of MMC on the morphology of activated cells populating collagen lattices by scanning electron microscopy

Cells exposed to 1 mg/ml MMC

Lattice surface cell density appeared reduced compared to controls. Cells appeared non-viable.

Cells exposed to 0.2 mg/ml MMC

Cells on the surfaces of lattices appeared non-viable. Additionally, the collagen matrix appeared ‘puckered’.

Cells exposed to 0.1 mg/ml MMC

The majority of cells on the surfaces of lattices appeared non-viable.

Cells exposed to 0.01 mg/ml MMC

Cells exhibited a range of morphologies including rounded (presumably non-viable), stellate and bipolar cells. The lattice surface cell density appeared reduced compared to controls.
3.2.3.6 Effects of 5FU and MMC on Actin Distribution

One day after seeding, a number of cells in control activated collagen lattices exhibited cell surface stress fibres. Following this, the cells showed a diffuse cytoplasmic fluorescence with phalloidin, indicative of migrating cells (Pfeffer et al. 1980; Herman et al. 1981) days 2 to 7. All nonactivated cells displayed surface stress fibres, characteristic of stationary cells (Pfeffer et al. 1980; Herman et al. 1981), 1 day after treatment (Figure 3.19). By day 3 these stress fibres had disappeared, and the cells exhibited a more diffuse actin distribution. Exposure of activated collagen lattices to 5FU did not appear to affect actin distribution compared to controls. However, actin appeared to be diffusely distributed in lattices containing nonactivated cells treated with 5FU or MMC compared to the presence of stress fibres in matched controls. A number of cells in activated and nonactivated collagen lattices treated with 1mg/ml of MMC were fluorescent for 7-aminoactinomycin D only.

3.2.3.7 Long Term Effects of Antimetabolites on Collagen Contraction

Single five minute exposures to 5FU (0.25, 2.5 and 25mg/ml) significantly inhibited (p<0.05) the contraction of collagen, compared to controls, upto 36 days post treatment in both activated and nonactivated lattices (Figure 3.20). Unlike the results shown in Section 3.2.3.3 and 3.3.3.4, the long term inhibitory effects of 5FU appeared to be similar in both nonactivated and activated lattices. The long term effects of MMC were similar to those of 5FU, causing a significant inhibition of contraction compared to controls at all concentrations tested (Figure 3.20). Again, there appeared to be little difference between the long term inhibitory effects of MMC on activated lattices compared to their non-activated counterparts.
Figure 3.19: Effect of 5FU and MMC on the actin cytoskeleton of nonactivated human tenon’s capsule fibroblasts populating collagen lattices

Actin cytoskeleton (green), nuclei (red/orange). Scale bar = 25μm.
Cells populating control lattices initially exhibited actin stress fibres (arrowheads). Only exposure to high 5FU (25mg/ml) affected cell morphology and the cytoskeleton compared to controls. Cells exhibiting nuclear staining only (arrows) were noted in lattices exposed to high MMC (1mg/ml). Only exposure to low concentrations of MMC (0.01mg/ml) did not appear to affect cellular morphology or the actin cytoskeleton compared to controls.
Graphs show that both 5FU and MMC significantly (p<0.05) inhibited long term collagen contraction compared to controls. Unlike the short term effects of these antimetabolites on contraction (Figures 3.15 and 3.16), the long term anti-contractile effects of both 5FU and MMC did not appear to be dependent upon the initial state of cellular activation. Cells populating lattices exposed to 5FU appeared non-viable only at the highest concentration (25mg/ml) in nonactivated samples. MMC appeared to elicit its effects via cytotoxicity at concentrations above 0.001mg/ml as judged by cellular morphology (rounded up cells).
In summary, single five minute exposures to 5FU and MMC inhibited cell-mediated collagen contraction compared to controls. The degree of short-term inhibition (up to 7 days) was dependent upon the initial state of cellular activation; lattices seeded with nonactivated cells being inhibited to a greater extent than lattices seeded with activated cells. The viability and cellularity of lattices seeded with activated cells, unlike their nonactivated counterparts, were not significantly affected by exposure to high concentrations (25mg/ml) of 5FU. The fact that cells do not proliferate in this model (see Figures 3.16 and 3.16; also Section 1) and 5FU also has antimetabolic as well as antiproliferative effects, suggests that the inhibition of contraction without cell death may be due to the inhibition of production of a protein(s) required for contraction, or conversely the stimulation of a protein(s) that inhibits contraction.

Exposure to MMC had significant effects both on cell viability and cellularity in lattices seeded with activated or nonactivated cells, suggesting that the inhibition of contraction was due in full or part to cytotoxic effects. Exposure to 5FU or MMC also affected cellular morphology and actin distribution compared to controls. The inhibitory effects of both 5FU and MMC on cell-mediated collagen contraction were also found to be long term (upto 36 days), although these effects appeared to be less dependent upon the degree of cellular activation.
During this series of experiments it was noted in control lattices by haematoxylin staining of paraffin wax embedded sections and by SEM, that in addition to collagen reorganisation a series of extracellular spaces (holes / tunnels) were present in the lattices and that the matrix adjacent to the cells appeared to have been modified (Figure 3.21). These holes/tunnels were only apparent in the presence of cells, i.e., they were absent in areas of the lattices not in close proximity to the cells, or lattices prepared in the absence of cells. These observations taken in conjunction with the above findings, that 5FU in particular may be inhibiting collagen contraction via antimetabolic effects, suggested the possibility that reorganisation/remodelling of the matrix via enzymic activity (namely the matrix metalloproteinases; MMPs) may play a crucial role in the process of cell-mediated collagen contraction. As the contraction of collagen is regarded as resulting from the tractional forces exerted by cells migrating upon their substratum (see Section 1), it was hypothesised that the MMPs were involved in contraction by allowing cells to penetrate and migrate through their surrounding collagen matrix, resulting in the generation of tractional forces within the lattice and ultimately contraction of this lattice. The results of the investigations to test this hypothesis are described in the following Section (3.4)
Figure 3.21: Reorganisation and potential remodelling of the surrounding matrix by human tenon’s capsule fibroblasts within collagen lattices

Haematoxylin stained paraffin wax section day 1 post seeding

Haematoxylin stained paraffin wax section day 3 post seeding

Haematoxylin stained paraffin wax section day 7 post seeding

Scanning electron micrograph day 1 post seeding

Scanning electron micrograph day 3 post seeding

Scanning electron micrograph day 7 post seeding

Key
Small arrows indicate lattice reorganisation / modification.
Large arrows indicate appearance of 'normal' lattice.
Arrowheads indicate holes / tunnels within the matrix.

Scale bars in paraffin sections = 50µm.
Scale bars for SEM shown on photographs. Ba = 40µm in inset.

Collagen matrix appeared to be reorganised/modified around cells. A series of holes/tunnels were apparent, associated with cells. The number of these holes/tunnels appeared to increase over the 7 day assay period.
3.3.1 Production of MMPs and TIMP During Collagen Contraction

Using QCRT-PCR, by 9 hours post seeding into collagen lattices cells produced markedly increased levels of mRNA for MMPs 1, 2 and 3 compared to monolayer cultures (Figure 3.22 and Table 3.2). These levels continued to increase further by day 1 post seeding but decreased by day 7, when contraction of the lattices had virtually ceased. The levels of MMP 9 were <1 copy/10^5 cells both in monolayer and in collagen lattices throughout the 7 day period. To determine if these increases in mRNA resulted in an increase in secreted MMP proteins, samples of conditioned media were analysed initially by gelatin zymography.

Cells seeded within collagen, when actively contracting these lattices, produced four gelatinolytic species into the overlying media (57, 72, 91 and 100kD). Two of these species (57 and 72kD) increased in quantity over the 7 day period (Figure 3.23). The same profile was seen from samples of homogenised lattices. Upon incubation with aminophenylmecuric acetate (APMA) which allows the autocatalytic cleavage of proenzymes (Stricklin et al. 1983), a reduction in the 100 and 72 kD (MMP 2) species was seen, with production of a 62 kD (active MMP 2) species and an increase in the levels of the 57 kD (MMPs 1 and 3) species (Figure 3.23). The production of gelatinolytic activity during collagen contraction was also observed for fibroblasts seeded onto the surface of lattices (Figure 3.23), and was similar to that seen for cells seeded within collagen (note that volumes were corrected for cell number). In order to determine if the gelatinolytic
activities produced during collagen contraction were in fact MMPs, samples of day 7 conditioned medium were incubated with 1, 10-phenanthroline, which has been reported previously to specifically inhibit MMP activity by chelating Zn\(^{2+}\) (Le et al. 1991; Galis et al. 1995). Upon incubation with increasing concentrations of 1,10-phenanthroline, a reduction in the amount of gelatinolytic activity in samples was seen (Figure 3.23). These findings were true regardless of whether samples of conditioned medium from cells in or on lattices, or homogenised lattices were used.

This suggests that the gelatinolytic activity produced during contraction of collagen lattices by cells within or upon the lattices were in fact MMPs. Although gelatin zymography gives information on molecular weight and whether the active species are in pro- or active forms, it only allows semi-quantitation of the amount of activity present in samples. Therefore, the levels of MMPs 1, 2, 3 and TIMP 1 were quantitated using specific ELISAs.

The levels of MMPs 1, 2, 3 and TIMP 1 in samples of conditioned medium from cells seeded within and contracting collagen lattices all increased over the 7 day assay period (Table 3.3). In particular, MMPs 2 and 3 appeared to increase to the greatest degree followed by MMP 1 and TIMP 1, which increased to a similar degree.
Figure 3.22: Analysis of MMP and TIMP mRNA expression by human tenon's capsule fibroblasts in monolayer and within collagen lattices by QCRT-PCR

Montage shows MMP/TIMP expression in fixed quantities of total RNA (1μg/lane). Levels of MMP and TIMP expression were quantitated, corrected for RNA recovered and cell number for each sample. Results showed that MMPs 1, 2 and 3 were markedly upregulated during culture within and during contraction of collagen lattices compared to monolayers (see Table 3.3). TIMP 1 levels did not differ greatly between monolayer and collagen lattices (see Table 3.3). Top set of bands in each panel indicate amplified message from cell sample, while the bottom set of bands indicate amplified synthetic template. See Figure 2.3 for an overview of the technique.
Table 3.2: Quantitation of MMPs and TIMP mRNA produced during collagen contraction, by QCRT-PCR

<table>
<thead>
<tr>
<th>Message</th>
<th>mRNA Copy Number / 10^3 Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Monolayer</td>
</tr>
<tr>
<td>MMP 1</td>
<td>5</td>
</tr>
<tr>
<td>MMP 2</td>
<td>NQ</td>
</tr>
<tr>
<td>MMP 3</td>
<td>NQ</td>
</tr>
<tr>
<td>MMP 9</td>
<td>NQ</td>
</tr>
<tr>
<td>TIMP 1</td>
<td>89</td>
</tr>
</tbody>
</table>

NQ = not quantitatable

Table 3.3: ELISA quantitation of MMPs and TIMP proteins produced in samples of conditioned medium by tenon's capsule fibroblasts contracting collagen lattices

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean Protein Concentration (ng/ml; +/-S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMP 1</td>
</tr>
<tr>
<td>1</td>
<td>4.128 (0.021)</td>
</tr>
<tr>
<td>3</td>
<td>5.635 (0.012)</td>
</tr>
<tr>
<td>7</td>
<td>7.956 (0.270)</td>
</tr>
</tbody>
</table>

Tables show that both mRNA and protein levels were markedly upregulated during culture within and during the contraction of collagen lattices.

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Figure 3.23: Analysis and inhibition of MMP protein activity produced during human tenon’s capsule fibroblast mediated collagen contraction, by gelatin zymography

A. MMP activity produced, by cells seeded within lattices, in samples of conditioned medium

Lane 1 = day 1 post seeding  
Lane 2 = day 3 post seeding  
Lane 3 = day 7 post seeding

Photograph shows gelatinolytic activity increased over the 7 day contraction assay. Four bands of activity were produced, having M, of 100, 91, 72 and 57 kD.

B. Effects of APMA on MMP profiles produced by cells within collagen lattices

Lane 1 = day 1 post seeding  
Lane 2 = day 3 post seeding  
Lane 3 = day 7 post seeding

Photograph shows 100, 91 and 72kD activities were reduced upon incubation with APMA. This suggested these activities were proenzymes. The 57kD activity was not affected by APMA treatment, indicating this was an active form species. The presence of a 65kD species following APMA treatment was noted.

C. MMP activity produced, by cells seeded upon lattices, in samples of conditioned medium

Lane 1 = day 1 post seeding  
Lane 2 = day 3 post seeding  
Lane 3 = day 7 post seeding

Photograph shows a gelatinolytic profile similar to that seen for cells seeded within matrix. As for cells within the matrix, the gelatinolytic activity produced by cells seeded upon the matrix increased over the 7 day contraction assay.

D. Inhibition of MMP activity produced by cells within collagen lattices using 1, 10-phenanthroline

Lane 1 = Developing buffer only  
Lane 2 = 2nM phenanthroline in developing buffer  
Lane 3 = 200nM phenanthroline in developing buffer  
Lane 4 = 20μM phenanthroline in developing buffer  
Lane 5 = 2mM phenanthroline in developing buffer

Photograph shows incubation with increasing concentrations of phenanthroline reduced the activity of gelatinolytic species produced during contraction. This suggested that the gelatinolytic activities present in conditioned medium were MMPs.
A. 

Lane 1 2 3

Mr

100 kD
91 kD
72 kD
57 kD

B. 

Lane 1 2 3

72 kD
65 kD
57 kD

C. 

Lane 1 2 3

100 kD
91 kD
72 kD
57 kD

D. 

Lane 1 2 3 4 5
In summary, it appears that the production of MMPs and TIMP are markedly upregulated at both the mRNA and protein levels during collagen contraction. In addition, it appears that regardless of whether cells are seeded within or upon a collagen matrix, that MMP protein production is upregulated during collagen contraction. What was not clear from this set of experiments was whether the marked increase in MMPs/TIMP production were essential to the process of collagen contraction, or were produced as a consequence of contact with a collagen matrix. In addition to this, it was unclear whether the requirement for MMP/TIMP activity for collagen contraction differed between 2-dimensional (cells on) and 3-dimensional (cells in) cell-matrix interactions.

### 3.3.2 Effects of MMP Inhibitors on Collagen Contraction

To determine if this increased cellular derived MMP/TIMP activity upon culture on top of or within collagen lattices was required for the process of contraction, rather than being just a result of contact with collagen, the effects of three different broad spectrum, potent hydroxamic acid derived MMP inhibitors (Galardin™-MPI, BB-94 and CellTech) on contraction were investigated.

Exposure of lattices containing $5\times10^5$ cells (within the lattice) to Galardin™-MPI, BB-94 or CellTech resulted in a significant inhibition ($p<0.05$) of contraction day 7 post seeding compared to controls (Figure 3.24). The inhibitory effects appeared to be compound dependent with Galardin™-MPI and BB-94 causing significant inhibition ($p<0.05$) of contraction at concentrations of 100nM and above, while CellTech only significantly inhibited ($p<0.05$) contraction at concentrations of 10μM and above. The anti-contractile effects of all of these MMP inhibitors appeared to be cell number...
dependent, all compounds eliciting an increased inhibitory effect on the contraction of lattices seeded with $1\times10^5$ cells (Figure 3.24), compared to their counterparts seeded with $5\times10^5$ cells. Using this lower seeding cell density resulted in Galardin™-MPI, BB-94 and CellTech significantly ($p<0.05$) inhibiting contraction at concentrations of $1\text{nM}$, $1\text{nM}$ and $10\text{nM}$ respectively. It therefore appears that MMP inhibitors significantly reduce the degree of collagen contraction by cells seeded within a collagen matrix.

It was noted during this series of experiments by phase contrast microscopy that cells in lattices exposed to MMP inhibitors only produced small cytoplasmic processes into their surrounding matrix. This was unlike control lattices, where cells after initially producing cytoplasmic processes proceeded to spread assuming a stellate morphology, followed by further cell spreading and elongation with adoption of a mixed bipolar and elongated stellate morphology (see Section 3.3.4 for a more in depth analysis).

Unlike collagen contraction by cells seeded within the lattice, exposure of lattices with $7.5\times10^4$ cells upon their surface to Galardin™-MPI, BB-94 or CellTech did not significantly inhibit ($p>0.05$) contraction at day 7 post seeding compared to controls (Figure 3.25). Also in contrast to cells seeded within lattices, exposure to MMP inhibitors did not appear to affect the ability of these cells to spread upon the collagen matrix compared to controls, by phase contrast microscopy.
Graphs show that exposure to MMP inhibitors significantly (p<0.05) inhibited collagen contraction compared to controls. The anti-contractile effects of MMP inhibitors appeared to be cell number dependent, a reduced seeding cell density resulting in an increased inhibition of contraction for the same concentration of inhibitor. The degree of inhibition of contraction for individual inhibitors appeared to decrease in the order of Galardin™-MPI >BB-94 >CellTech. In every case where MMP inhibitors affected contraction, the cells appeared morphologically viable. It was noted however that exposure to MMP inhibitors, in addition to inhibiting contraction, appeared to reduce the ability of cells to spread and elongate within the matrix compared to controls.
Graphs show that exposure to three different MMP inhibitors did not significantly (p<0.05) affect the contraction of collagen lattices by cells seeded upon the lattice surface. Unlike cells within lattices MMP inhibitors did not affect the ability of cells to spread, or their morphology, compared to controls when cells were seeded on the lattice surface.
It therefore appears from these results that although cells seeded upon or within a collagen matrix both contract the matrix, which is associated with increased MMPs/TIMP production, it is only when cells are surrounded by matrix (cells in) that this MMP activity appears to be required for contraction of the matrix. In addition to this, these results also suggest that contact with a collagen matrix even in 2-dimensions (cells on) is sufficient to upregulate MMPs/TIMP activity, regardless of whether or not it is required for collagen contraction.

To further investigate the effects of MMP inhibition on collagen contraction by cells entrapped within this matrix, human recombinant TIMP 1 (in the overlying medium) and antibodies to MMPs 1, 2 or 3 (incorporated into the lattice) were used. All of these proteins significantly inhibited (p<0.05) collagen contraction compared to matched controls by day 7 post-seeding (Figure 3.26) although less effectively than the hydroxamic acid-derived inhibitors. As for the other MMP inhibitors, exposure to TIMP 1 or antibodies to MMPs 1, 2 or 3 did not appear to affect cell viability by morphological analysis using phase contrast microscopy. However as noted earlier, an inhibition of collagen contraction was associated with the inability of cells to spread within the collagen matrix and assume similar changes in morphology as controls.

The long term anti-contractile effects of Galardin™-MPI were also investigated by the addition of this inhibitor to contracting lattices and the effects of its removal from inhibited lattices (cells seeded within the matrix only). Removal of Galardin™-MPI from inhibited collagen lattices resulted in the continued contraction of lattices up to 48 days post-seeding (Figure 3.27), without apparent cell death. Addition of Galardin™-MPI to
contracting lattices was found to significantly inhibit contraction up to 48 days post
seeding compared to controls (Figure 3.27), again without apparent cell death.

In summary, it appears that MMP/TIMP activity is upregulated upon contact with
and during contraction of a collagen matrix. Also, MMP/TIMP activity only appears to be
required for the contraction of collagen when the cells are surrounded by matrix. Several
MMP inhibitors are capable of eliciting varying degrees of anti-contractile effects. The
degree of contraction occurring appears to be dependent upon the inhibitor used, its
concentration and the lattice cell number. Additionally the degree of contraction can be
partially controlled upon the addition of MMP inhibitor to contracting lattices, or its
removal from the system.

Although all of the above results strongly suggest a role for MMPs in collagen
contraction, the possible non-specific anti-contractile effects of the inhibitors used could
not be ruled out at this stage. The specificity of the actions of these inhibitors were
therefore further investigated, as described in the following Section (3.3.3).
Figure 3.26: Effects of TIMP 1 and polyclonal MMP antibodies on human tenon's capsule fibroblast mediated collagen contraction

Graphs show that exposure to human recombinant TIMP 1 or polyclonal antibodies to MMPs 1, 2 or 3 significantly (p<0.05) reduced collagen contraction by cells seeded within the matrix, although the anti-contractile effects were not as marked as for MMP inhibitors (see Figure 3.24). The inhibition of contraction was, like for MMP inhibitors, associated with a decreased ability of the cells to spread/penetrate their surrounding matrix. Cells in lattices exposed to TIMP 1 or antibodies appeared morphologically viable throughout the assay.
Figure 3.27: Effects of the removal and addition of Galardin™-MPI on collagen lattice contraction by human tenon’s capsule fibroblasts

Graphs show that anti-contractile effects of Galardin™-MPI could be partially reversed upon removal of the inhibitor, or induced early on in the course of contraction. There was no evidence of cell death within the lattices.
3.3.3 Specificity of the Effects of MMP inhibitors on Collagen Contraction

To eliminate the possibility that the MMP inhibitor effects on contraction were due to non-specific actions upon cellular functions essential to collagen contraction, their effects on RNA synthesis, cell number, cell viability, the actin cytoskeleton, attachment to collagen, and the inhibition of MMP activity produced during collagen contraction, were investigated.

Continual exposure to even the highest concentration of Galardin™-MPI did not adversely affect RNA synthesis, long term cell number or cell viability up to 48 days post-seeding in monolayer culture compared to controls (Figure 3.28). In addition to this, exposure of cells within collagen lattices to Galardin™-MPI did not appear to have significant cytotoxic effects as shown by LDH levels in the medium overlying lattices (Table 3.4) or the total LDH levels recovered from cells within lattices (Table 3.5), compared to controls up to 7 days post-seeding. These data, on the lack of cytotoxic effects of Galardin™-MPI on ocular fibroblasts, are further corroborated from the data in Section 3.3.2. This data indicated that cells within lattices exposed to MMP inhibitors appear morphologically viable throughout the assay. Secondly, exposure of cells seeded upon collagen matrices to Galardin™-MPI had no significant effect upon the degree of contraction compared to controls (Figure 3.25). If Galardin™-MPI had elicited cytotoxic effects, a concentration dependent inhibition of contraction of these lattices, with associated morphological changes of the cells, would have been expected. It therefore appears that Galardin™-MPI did not elicit its anti-contractile effects by reducing cell number or viability.
Graphs show exposure to Galardin™-MPI did not significantly (p>0.05) affect long term cell number or viability in monolayer culture compared to controls. Additionally, exposure to Galardin™-MPI did not affect the ability of cells to spread or their morphology.
Table 3.4: Effects of Galardin™-MPI on LDH release into overlying culture medium by cells populating collagen lattices

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean Absorbance at 492nm (+/- S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>0.351 (0.018)</td>
</tr>
<tr>
<td>3</td>
<td>0.349 (0.029)</td>
</tr>
<tr>
<td>7</td>
<td>0.407 (0.042)</td>
</tr>
</tbody>
</table>

Table 3.5: Effects of Galardin™-MPI on LDH release from cells populating collagen lattices

<table>
<thead>
<tr>
<th>Day</th>
<th>Mean Absorbance at 492nm (+/- S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>1.531 (0.028)</td>
</tr>
<tr>
<td>3</td>
<td>1.034 (0.026)</td>
</tr>
<tr>
<td>7</td>
<td>0.867 (0.032)</td>
</tr>
</tbody>
</table>

Tables show that exposure to Galardin™-MPI did not significantly (p>0.05) affect LDH release into overlying culture medium or the levels of LDH recovered from lattices, compared to controls. These results suggested Galardin™-MPI was not cytotoxic.
Contraction has been shown to be dependent upon an intact actin cytoskeleton and the ability of cells to attach to their matrix (see Section 1). To establish if the anti-contractile effects of Galardin™-MPI were due to non-specific effects on these parameters, the effects of Galardin™-MPI on these was investigated. Differences both in the distribution of actin and cell morphology within collagen lattices were seen upon exposure to Galardin™-MPI (Figure 3.29 C/D). To establish if this was due to direct cytoskeletal effects of Galardin™-MPI or differences in cell spreading and shape within the lattices, cells were exposed to the inhibitor in monolayer culture and the cytoskeleton stained. In monolayer culture Galardin™-MPI did not affect the ability of cells to spread, cellular morphology or the cytoskeleton compared to controls (Figure 3.29 E/F). This suggested that the differences seen within collagen lattices were due to differences in cell shape only.

Using a collagen type I attachment assay, exposure to Galardin™-MPI, BB-94 or CellTech MMP inhibitors did not significantly affect (p>0.05) the ability of cells to attach to a collagen matrix up to 24 hours post-seeding (Table 3.6 and Figure 3.29). By transmission electron microscopy, Galardin™-MPI was also shown not to significantly affect cellular attachment to the surrounding matrix compared to controls, as similar numbers of collagen fibres were seen associated with the cell surface (Figure 3.29). This inability of MMP inhibitors to prevent ocular fibroblast attachment to a collagen matrix is further supported by data in Figure 3.25, which shows that cells seeded onto the top of collagen lattices in the presence of MMP inhibitors were not significantly affected in their ability to contract this matrix compared to controls.
Figure 3.29: Effects of Galardin™-MPI on cellular functions essential to collagen contraction

A. Degree of collagen contraction day 7 post seeding (control)
B. Degree of collagen contraction day 7 post seeding (+ Galardin)

C. Actin cytoskeleton within collagen lattice (control)
D. Actin cytoskeleton within collagen lattice (+ Galardin)

E. Actin cytoskeleton in monolayer culture (control)
F. Actin cytoskeleton in monolayer culture (+ Galardin)

G. Transmission electron micrograph of cellular attachment to collagen (control)
H. Transmission electron micrograph of cellular attachment to collagen (+ Galardin)

I. Attachment of cells to collagen / adhesion assay (control)
J. Attachment of cells to collagen / adhesion assay (+ Galardin)

Key
A./B. Arrows/arrowheads indicate degree of contraction. Scale bars = 8mm.
C./D. Arrows indicate actin stress fibres. Scale bar = 25μm (10μm inset).
E./F. Arrows indicate actin stress fibres. Scale bar = 25μm.
G./H. Arrowheads indicate cellular attachment to collagen, CM indicates collagen matrix. Scale bars = 2μm.
I./J. Scale bar = 25μm.

Photographs show that Galardin™-MPI inhibited contraction and did not significantly affect the cytoskeleton or attachment of cells to a collagen matrix, compared to controls. The differences in the cytoskeleton seen in C./D. appeared to be due to differences in cellular morphology, as Galardin™-MPI did not affect either morphology or the cytoskeleton in monolayer cultures compared to controls (see E./F.).

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Table 3.6: Effects of MMP inhibitors on the ability of tenon's capsule fibroblasts to attach to a collagen type I matrix

<table>
<thead>
<tr>
<th>Hours Post Seeding</th>
<th>Mean Absorbance at 570nm (+/- S.E.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Galardin™-MPI</td>
</tr>
<tr>
<td></td>
<td>0 100nM 10µM 100µM 100nM 10µM 100µM 100nM 10µM 100µM</td>
</tr>
<tr>
<td>1</td>
<td>0.316 (0.058) 0.288 (0.031) 0.334 (0.029) 0.313 (0.021) 0.310 (0.032) 0.309 (0.020) 0.307 (0.035) 0.307 (0.042) 0.281 (0.020) 0.316 (0.044)</td>
</tr>
<tr>
<td>24</td>
<td>0.512 (0.028) 0.483 (0.017) 0.527 (0.031) 0.531 (0.026) 0.507 (0.039) 0.493 (0.027) 0.486 (0.034) 0.503 (0.019) 0.527 (0.041) 0.531 (0.015)</td>
</tr>
</tbody>
</table>

Table shows that the MMP inhibitors did not significantly (p>0.05) affect the ability of cells to attach to a collagen type I matrix compared to controls. Additionally, they did not affect the morphology or the ability of cells to spread upon the collagen matrix compared to controls (also see Figure 3.29)
The ability of the MMP inhibitors used in this study, which were shown to inhibit collagen contraction to varying degrees, to inhibit MMP activity produced during collagen contraction was investigated. By zymography, exposure to Galardin™-MPI in culture medium abolished only the 57kD MMP activity seen from actively contracting lattices. This appeared to be due to dissociation of the inhibitor from MMP protein during zymography, as all activity was increasingly abolished when increasing concentrations of Galardin™-MPI (0.1 nM - 100 μM) were added to the developing buffer (Figure 3.30). In addition to this, addition of increasing concentrations (0.1 nM - 100 μM) of either BB-94 or CellTech also increasingly inhibited MMP activity produced during collagen contraction, by gelatin zymography (Figure 3.30). It was noted that these MMP inhibitors qualitatively inhibited the MMP activity in culture medium, from contracting collagen lattices, in the order of Galardin™-MPI > BB-94 > CellTech. Interestingly as shown in Figure 3.24 Galardin™-MPI and BB-94 appeared to be more potent inhibitors of collagen contraction than CellTech, suggesting that their differences in ability to inhibit collagen contraction may be due to their differential inhibition of MMP activity.
Figure 3.30: Effects of MMP inhibitors on MMP activity in conditioned medium produced during collagen contraction, by gelatin zymography

Photographs show that exposure to increasing concentrations of MMP inhibitors reduced MMP activity present in samples of 7 day conditioned medium from contracting collagen lattices.
From these results it therefore appears that the MMP inhibitors elicit their anti-contractile effects by specifically inhibiting MMP activity and not by non-specific effects on RNA synthesis, cell number, cell viability, the cytoskeleton or attachment to collagen.

3.3.4 Role of MMPs and TIMP in Collagen Contraction

During this study it was noted that during collagen contraction, cells within the matrix underwent a series of morphological changes. However upon exposure to MMP inhibitors, which inhibited collagen contraction, cells populating these lattices appeared viable and to have a morphology associated with the 'early' culture within a matrix. These cells retained this morphology throughout the collagen contraction assay.

As the process of collagen contraction is regarded as resulting from the tractional forces exerted by cells migrating upon their substratum (see Section 1), the effects of MMP inhibitors on this migratory process and cell morphology during collagen contraction were investigated using time lapse video. By time lapse video, cells contracting collagen in this model undergo an ordered series of morphological changes from an initial spherical appearance post-seeding, followed by cell spreading with the production of cytoplasmic processes (within 2 hours post-seeding), a stellate appearance (24 hours) and finally adoption of a mixed stellate and bipolar morphology (24 hours onwards) which is accompanied by movement through the collagen lattice (shown schematically in Figure 3.31 and by time lapse video in Figure 3.32). The processes of cell spreading, adoption of a stellate or bipolar morphology and movement through the collagen lattice all require the cell to penetrate into its relatively dense (5mg/ml collagen) surrounding matrix.
Significantly, cells exposed to MMP inhibitor (Figure 3.32) exhibited a decreased ability to spread/penetrate into the surrounding matrix compared to controls. This decrease in cell spreading/penetration of ocular fibroblasts was always accompanied by inhibition of both contraction and the movement of these cells through the matrix. The inhibition of MMP activity by Galardin™-MPI in the absence of a surrounding collagen matrix (i.e., in monolayer culture) had no effect on the ability of these cells to spread, elongate and migrate upon their substratum compared to controls (Figure 3.33). In addition to this, exposure of cells in monolayer culture to the MMP inhibitors BB-94, CellTech, TIMP 1 or antibodies to MMPs 1, 2 or 3 did not significantly affect the ability of cells to spread and elongate in monolayer culture compared to controls (Figure 3.34).
Figure 3.31: Schematic diagram of fibroblast morphology during collagen contraction

- Rounded cells
- Small cytoplasmic processes
- Further cell spreading, stellate morphology
- Cells elongate, mixed bipolar and stellate morphology

0 Hours
2 Hours
24 Hours
24+ Hours
Figure 3.32: Effects of Galardin™-MPI on human tenon's capsule fibroblast spreading and movement in collagen lattices by time lapse video

Time post seeding into lattices is shown in the left hand corner of each photograph.
Arrow indicates cell of interest
Arrowhead indicates original position of this cell

Photographs show that exposure to Galardin™-MPI inhibited the spreading / penetration of cells into the surrounding matrix, in addition to inhibiting the subsequent movement of these cells through the matrix compared to controls.
Figure 3.33: Effects of Galardin™-MPI on human tenon’s capsule fibroblast spreading and movement in monolayer culture by time lapse video

Time post seeding into monolayer is shown in the left hand corner of each photograph.
Arrow indicates cell of interest
Arrowhead indicates original position of this cell

Photographs show that exposure to Galardin™-MPI did not inhibit the spreading or movement of these cells upon tissue culture plastic compared to controls.
Figure 3.34: Effects of MMP inhibitors on human tenon’s capsule fibroblast morphology in monolayer culture

Photographs show exposure to MMP inhibitors (BB-94 and CellTech), TIMP 1, or MMP antibodies did not affect the ability of cells to spread or their morphology compared to controls. Additionally, cells appeared to proliferate and remain viable throughout the culture period.

Scale bar = 25 μm.
These results suggest that the MMPs are involved in allowing cells that are surrounded by a collagen matrix firstly to spread, then elongate and migrate through this collagenous matrix. It appears that these processes of cell spreading, elongation and migration result in the generation of tractional forces within the collagen matrix and the subsequent contraction of this matrix.

To determine if this requirement for MMP activity was not just specific to the fibroblast type (human Tenon's capsule), the effects of MMP inhibition on contraction by fibroblasts from other tissue sites and species including: human corneal; human dermal; rat dermal; rat synovial sheath; rat endotendon; and rabbit conjunctival fibroblasts, were also investigated. Exposure to Galardin™-MPI resulted in a significant (p<0.05) inhibition of contraction in all of the cell types tested (Figure 3.35). By time lapse video, the inhibition of contraction for all of these fibroblasts was also accompanied by a decreased cellular invasion into and movement through the matrix as seen for human Tenon's capsule fibroblasts. This suggests that the requirement of MMP activity for cell spreading, elongation and movement through a collagenous matrix, the resultant generation of tractional forces and ultimately the contraction of collagen may be ubiquitous throughout different tissue sites and species.
Figure 3.35: Effects of Galardin™-MPI on collagen contraction by fibroblasts from different tissue sites and species

Graphs show that exposure to Galardin™-MPI significantly (p<0.05) inhibited collagen contraction regardless of the tissue site or species the fibroblasts were derived from. Inhibition of contraction was accompanied by a reduced ability of cells to penetrate/spread into their surrounding matrix. All cells appeared viable by morphological assessment (also see Figure 3.32).
In summary it appears that the production of MMPs/TIMP are markedly upregulated at both the mRNA and protein levels during the contraction of collagen lattices, regardless of whether cells are seeded within or upon the matrix. Exposure of collagen lattices, containing cells, to a number of MMP inhibitors resulted in a significant inhibition of collagen contraction compared to controls.

As shown earlier in Sections 3.3.3 and 3.3.4, exposure to the antimetabolites 5FU and MMC at lower concentrations significantly inhibited cell-mediated collagen contraction by activated cells, without causing cell death. These results suggested that this inhibition of contraction may have been due to the inhibition of a protein(s) required for contraction, or conversely the stimulation of a protein(s) that inhibited contraction. In view of the above results, it was hypothesised that the anti-contractile effects of single, five minute exposures to MMC and particularly 5FU may have been due in full or part to their antimetabolic effects of reducing overall MMP activity. The results of investigations to test this hypothesis are given below.

3.4.6 Effects of Antimetabolites on MMP and TIMP Protein Production by Cells

Populating Collagen Lattices

Upon exposure to 25mg/ml 5FU, a significant reduction (p<0.05) in the levels of MMPs 1, 2 and TIMP 1 were seen (Figures 3.36). In contrast, a significant increase (p<0.05) in the levels of MMP 3 production was observed. At lower concentrations of 5FU (0.25 and 2.5mg/ml), only the levels of MMPs 1 and 3 were significantly elevated (p<0.05) by day 7 compared to controls, exposure to 2.5mg/ml eliciting a greater effect. It therefore appears that high concentrations of 5FU significantly reduce the levels of MMPs
1, 2 (almost completely) and TIMP 1, but not MMP3. Lower concentrations of 5FU either markedly stimulate MMP protein production (MMPs 1 and 3) or have no real effect compared to controls (MMP 2 and TIMP 1), resulting in an overall net increase in MMP production during collagen contraction.

Similarly to 5FU, exposure to high concentrations of MMC (0.1mg/ml) significantly reduced (p<0.05) the production of MMPs 1, 2, 3 and TIMP 1 by day 7 compared to controls (Figure 3.37). Exposure to lower concentrations of MMC (0.01 and 0.001mg/ml), like those for 5FU, significantly stimulated (p<0.05) MMP 1 and MMP 3 production compared to controls. Unlike the effects of 5FU, exposure to lower concentrations of MMC significantly inhibited (p<0.05) MMP 2 production, while TIMP 1 levels appeared unaffected. As for 5FU, the reduction in MMP levels detected by ELISA following exposure to high levels of MMC may account for the inhibition of contraction seen upon exposure to this agent. However, exposure to lower MMC concentrations appeared to stimulate an increase in overall MMP production. However, the ELISA techniques used in this study give no indication of the actual biological activity of the proteins detected. Therefore, the biological MMP activity of these 5FU and MMC samples was also investigated by gelatin zymography.
Graphs show that exposure to high concentrations (25mg/ml) of 5FU significantly (p<0.05) reduced the production of MMPs 1, 2 and TIMP 1 during collagen contraction compared to controls. Exposure to lower concentrations of 5FU either stimulated (MMPs 1 and 3), had no significant (p>0.05) effect (MMP 2 / TIMP 1) or significantly (p<0.05) reduced (TIMP 1 for 2.5mg/ml 5FU) the levels of these proteins over the 7 day assay period. As for controls the levels of MMPs and TIMP 1 produced increased over the 7 day assay period regardless of treatment. Cells within lattices appeared viable, as judged by morphology, throughout the assay.
Figure 3.37: Effects of the antimetabolite MMC on MMP and TIMP production by ELISA, during human tenon's capsule fibroblast mediated collagen contraction

Graphs show that exposure to high concentrations of MMC (0.1 mg/ml) significantly (p<0.05) reduced the levels of all MMPs and TIMP 1 produced during the assay, compared to controls. Exposure to low concentrations of MMC either stimulated (MMPs 1 and 3), reduced (MMP 2) or had no real effect (TIMP 1) on the levels of MMPs and TIMP 1 produced during the 7 day assay period. As for 5FU experiments (see Figure 3.36), all cells within lattices appeared viable throughout the 7 day period of the assay.
Exposure to all concentrations of 5FU resulted in the production of increasing amounts of MMP activity days 1 to 7 post seeding into collagen lattices (Figure 3.38). On a normalised volume per volume basis (to allow direct comparison of activities), the relative degree of MMP activity in each 5FU treated sample suggested a concentration dependent decrease in MMP biological activity, with increasing concentrations of 5FU. Additionally, no difference was seen in the MMP profile between samples. It therefore appears that unlike MMP protein quantitation by ELISA, 5FU decreases the actual amount of MMP biological activity produced during collagen contraction in a concentration dependent manner.

Similar results were observed upon analysis of samples of conditioned medium from collagen lattices exposed to MMC. Differences in the MMP profiles were noted between samples, in addition to a MMC concentration dependent reduction (lower MMC concentrations resulting in higher levels of production of biological MMP activity) compared to PBS controls.
Figure 3.38: Effects of exposure to MMC and 5FU on the biological activity of MMPs produced by human tenon's capsule fibroblasts during collagen contraction, by gelatin zymography

Photographs show the effects of antimetabolites on MMP production in samples of conditioned medium collected days 1, 3 and 7 (d1, d3, d7) during collagen contraction. Exposure to MMC (0.1 and 0.01mg/ml) altered the MMP profile produced during collagen contraction. Exposure to both MMC (0.1 - 0.001mg/ml) or 5FU (only 25 and 2.5mg/ml) resulted in a decreased production of MMP biological activity during contraction, compared to controls. The reduction in biological activity appeared to be dependent upon the concentration of antimetabolite used. Cells appeared viable throughout the 7 day conditioned medium collection period.
In summary, these results suggest that MMP biological activity produced during collagen contraction is reduced in a concentration dependent manner upon exposure to the antimetabolites 5FU and MMC compared to PBS controls. It therefore appears from ELISA and zymography data that upon exposure to antimetabolites that even though MMP protein production is markedly upregulated during collagen contraction, the actual amounts of biologically active MMPs are in fact reduced. Finally, data from this study shows that both 5FU and MMC inhibited collagen contraction (Section 3.2.3), that MMP activity is essential to this contractile process (Section 3.3) and both 5FU and MMC result in a reduced production of MMP biological activity during collagen contraction. Although this data indicates that 5FU and MMC may be eliciting their anti-contractile effects by reducing cellular derived MMP biological activity, it is possible that these effects either only contribute partially to the overall anti-contractile effects or that they occur as a result of some other as yet unidentified inhibitory mechanism(s).
3.4 Summary of Results


Growth Factor and Growth Factor Receptor Expression

Exposure to the antimetabolites 5FU and MMC initially reduced the levels of TGFβ1 mRNA compared to controls. This was followed by a continued reduction in mRNA levels or an increase followed by a sustained decrease to controls levels, by day 48 post-exposure. The production of TGFβ1 and bFGF growth factor proteins appeared to either peak in excess of controls days 3-14 followed by a sustained decrease towards controls, or remain elevated for the 36-48 days compared to controls. Exposure to 5FU generally reduced bFGF receptor (protein), TGFβ type II receptor and EGF receptor (mRNA and protein), with cells fully or partially recovering expression to that of controls by day 48. Exposure to MMC appeared to elicit less marked inhibitory effects than those of 5FU, with cells either recovering expression similar to controls or in some cases exceeding control levels.

Extracellular Matrix Molecule Production

Single, five minute exposures to 5FU or MMC significantly reduced the production of collagen type I and fibronectin, both at the mRNA and protein levels, throughout the 48 day assay period. However unlike collagen type I protein production, growth arrested cells appeared to partially recover the ability to produce fibronectin by day 48 post-exposure to antimetabolites. Exposure to high concentrations of 5FU (25mg/ml) appeared to result in a significantly elevated production of collagen type III mRNA production days 24-48,
compared to controls. However, this was not reflected in a subsequently elevated level of collagen type III protein production. Exposure to lower concentrations of 5FU (2.5 and 0.25mg/ml) and to MMC generally lowered collagen type III mRNA production. Although a peak of collagen type III protein production was observed for cells growth arrested by these agents at day 7 post-exposure, levels decreased towards those of controls over the remaining period of the assay.

B). **Effects of Antimetabolites on Cellular Aspects of Fibroblast Wound Healing Behaviour.**

**Proliferation and Migration**

Single, five minute exposures to high (25mg/ml) concentrations of 5FU induced growth arrest and significantly reduced the migratory ability of fibroblasts for up to 48 days. Lower concentrations also induced growth arrest and reduced migration for shorter periods, with a subsequently larger recovery of function. MMC appeared to induce growth arrest or significantly reduce proliferation in a concentration dependent manner, the inhibitory effects on migration being less pronounced with cells almost fully recovering their migratory capacity.

**ECM Contraction**

Single, five minute exposures to 5FU or MMC significantly inhibited both short (up to 7 days) and long term (up to 48 days) collagen contraction. Whereas the degree inhibition of short term contraction appeared to be dependent upon the initial state of cellular activation, the inhibition of long term contraction did not. MMC appeared to
inhibit contraction by a combination of cytotoxic and antimetabolic effects (depending upon the concentration of MMC used), while the effects of 5FU appeared to be elicited primarily via antimetabolic effects. Unlike for proliferation and migration, cells exposed to 5FU and MMC (except at 0.001mg/ml) did not regain their contractile ability.

**MMPs and ECM Contraction**

MMP activity appears to be an essential component of fibroblast-mediated collagen contraction, but only when the cells are surrounded by matrix. It seems that MMPs allow cells within the matrix to spread, penetrate and then move through this matrix. It is these processes of spreading, penetration and migration that generate the tractional forces within the matrix that subsequently result in contraction of the matrix. This requirement of MMP activity for collagen contraction was found to be true for fibroblasts derived from different tissue sites and species, suggesting it may be a ubiquitous mechanism. Finally, the anti-contractile effects of 5FU and MMC appear to result, at least in part, from their antimetabolic effects on the production of biologically active MMPs.
Chapter 4: DISCUSSION

4.1 Wound Healing and its Modulation

4.1.1 The importance of wound healing and the fibroblast

The wound healing process, in addition to restoring lost or damaged tissue, is a major cause of clinical morbidity throughout the human body. The healing process has been shown to be of particular importance in the eye, playing a major role in the pathogenesis or failure of treatment of many blinding or visually disabling conditions in the world today. Although the healing response is a complex process involving the interactions of several chemical mediators and cell types, the fibroblast has been identified as a key player. Fibroblasts perform several crucial functions including the expression of growth factors and their receptors, the production of ECM, proliferation, migration and ECM contraction. These aspects of cellular behaviour are regulated by several extracellular stimuli which, acting via cell surface receptors and intracellular signalling cascades, elicit a nuclear response and ultimately a change in cellular behaviour (Figure 4.1).

4.1.2 Modulation of wound healing by growth arrest

In view of the clinical problems associated with the wound healing response, particularly in the eye, much research has concentrated on its modulation. Several agents and their use in the modulation of ocular scarring, for example after glaucoma filtration surgery, have been studied. Many groups have investigated the use of antiproliferatives/antimetabolic agents, which were traditionally used in the treatment of cancer, primarily for their ability to inhibit cell division. As such, much of the antiscarring research in ophthalmology has concentrated on the use of these agents on the basis that reduced
cellular numbers present at the wound site, would result in a reduction of the healing response. Blumenkranz and colleagues established that the antimetabolite 5FU inhibited the proliferation of fibroblasts \textit{in vitro} and ocular scarring \textit{in vivo} (Blumenkranz et al. 1982; Blumenkranz et al. 1984). Following these findings, an antiscarring regimen involving subconjunctival 5FU injections after glaucoma filtration surgery was developed in Miami, Florida, U.S.A., resulting in a multicentre trial (The Fluorouracil Filtering Surgery Study Group. 1989). Largely unnoticed, Chen \textit{et al} (1990; 1983) had been using single applications of another antimetabolite, MMC, for more than a decade. Further research by Khaw \textit{et al} demonstrated that both 5FU and MMC had long term antiproliferative effects on fibroblasts, with single exposures as short as five minutes (Khaw et al. 1992c; Khaw et al. 1991b). This group subsequently showed that effective suppression of proliferation in excess of 36 days, without cell death, could be achieved (Khaw et al. 1992c; Khaw et al. 1992d). These single, five minute treatments were also found to be effective \textit{in vivo}, being titratable in terms of length of action (Doyle et al. 1993; Khaw et al. 1993b) and focal (Khaw et al. 1993a; Khaw et al. 1992a). It therefore appeared in many instances that antimetabolites were eliciting their antiscarring effects by causing growth arrest of fibroblasts, which resulted in a overall reduction in fibroblast proliferation at the wound site over the course of the healing process.
Extracellular stimuli including growth factors and ECM molecules interact with specific cell surface receptors (e.g., growth factor receptors, integrins). This results in signal transduction via a number of cascades to the nucleus. The subsequent nuclear response may result in changes in DNA replication, RNA and protein synthesis, ultimately producing a cellular response or change in behaviour.
4.1.3 The importance of growth arrest

The process of growth arrest induced by antimetabolites is important for several reasons. Firstly, the regulation of cell homeostasis, throughout the body, is thought to be via a combination of processes including proliferation, programmed cell death (apoptosis) and growth arrest (Marx, 1993; Williams, 1991). Secondly, single intraoperative, five minute exposures to 5FU and MMC are now the standard treatment for many patients undergoing glaucoma filtration surgery world-wide, and are currently the subject of a five year Medical Research Council clinical trial at Moorfields Eye Hospital (London). However, what is currently unclear is the overall effect of these single application treatments on fibroblast-mediated scarring. It may be that fibroblasts, although growth arrested, are capable of carrying out functions such as ECM contraction and ECM production which may still contribute to a healing response. These growth arrested fibroblasts may also still be able to interact with neighbouring cells, via the production of molecules such as growth factors, and influence the behaviour of other growth arrested cells or surrounding 'normal' cells. If growth arrested cells were still capable of carrying out several healing functions, this may contribute to the failure of such surgical procedures.

Without antimetabolite treatment, approximately 74% of patients who have failed previous surgery will fail repeat glaucoma filtration surgery (The Fluorouracil Filtering Surgery Study Group, 1996). There is also evidence to suggest that high risk patients who have received antimetabolites can still fail glaucoma filtration surgery due to scarring, although the overall failure rate is lower (51%; The Fluorouracil Filtering Surgery Study Group, 1996). In addition, human diploid epidermal keratinocytes are serially cultivated on
layers of growth arrested 3T3 fibroblast feeder layers, a technique routinely used in many laboratories. The original methods described by Rheinwald and Green for the production of these feeder layers included inducing growth arrest by radiation (Rheinwald and Green, 1975) or exposure to MMC (Rheinwald, 1980). Although these methods are still in use, over twenty years later, the exact mechanisms of how they support keratinocyte growth are unclear. A report by Blacker et al (1987), suggested that 3T3 fibroblasts were capable of producing eicosanoids (prostaglandin E₂ and 6-keto-prostaglandin F₁α) even though growth arrested. These molecules may contribute to the support of keratinocyte growth. It therefore appears that these growth arrested feeder layers do not just serve as an inert structural matrix for epidermal cell growth, but may produce molecules that influence their behaviour.

In summary, the healing response is a major cause of clinical morbidity throughout the human body. The fibroblast is the key player in this response, carrying out several crucial functions. The modulation of fibroblast proliferation via single, five minute exposures to antimetabolites has been shown to result in growth arrest in vitro, and in reduced scarring in vivo. However, following exposure to antimetabolites scarring is sometimes seen in vivo. Additionally, growth arrested fibroblast feeder layers are used to support epithelial cell growth. Both of these observations suggest that growth arrested fibroblasts may still be able to exhibit behaviour associated with wound healing. Whether this is true or not is currently unclear. However, elucidation of these issues would not only contribute to a greater understanding of the mechanisms underlying fibroblast-epithelial cell interactions, but may also have clinical implications with respect to the modulation of scarring in vivo.
The aim of this study was to investigate the effects of growth arrest induced by antimetabolites on molecular and cellular aspects of fibroblast wound healing behaviour. As illustrated in Figure 4.1, the control of fibroblast behaviour and the resultant cellular response can be divided into a number of aspects. The effects of antimetabolites on these aspects of behaviour, their relevance in the context of the current literature, possible mechanisms of action and their significance, are discussed below.

4.2 Growth Arrested Fibroblasts are Capable of Producing Regulatory Molecules

4.2.1 Growth factors and growth factor receptors

Results from experiments performed in this study indicated that although cells were growth arrested, they were capable of performing several molecular functions. The production of total RNA and protein by control cultures was found to decrease continually over the 48 day culture period. This was in contrast to cells exposed to antimetabolites which appeared to synthesise increasing amounts of RNA (peaking at day 14), followed by a subsequent maximal production of protein around day 24. Exposure to 5FU (25mg/ml) and MMC (0.1mg/ml) significantly reduced TGFβ1 mRNA production up to day 24, while exposure to lower concentrations of these agents resulted in a maximal production at day 14 followed by a decrease to control levels. The production of TGFβ1 and bFGF growth factor proteins by growth arrested cells (as EGF was not detectable at the mRNA or protein levels) exhibited a slightly different pattern, with production peaking between days 3-14 followed by a decrease towards control levels.
As illustrated in Figure 4.1, the regulation of fibroblast behaviour is via the actions of external stimuli that include growth factors. Both TGFβ and bFGF have been shown to stimulate a variety of fibroblast functions including proliferation, migration, ECM contraction and ECM synthesis (Khaw et al. 1994b; Assouline et al. 1992; Gospodarowicz, 1974; Ignotz et al. 1987; Edwards et al. 1988), as well as having effects on other cell types involved in the wound healing process including endothelial and epithelial cells. Therefore, it is possible that growth arrested fibroblasts may be able not only to influence the behaviour of surrounding growth arrested or normal fibroblasts, but also other locally resident cell types. TGFβ has also been shown to elicit an autoinductive effect on fibroblasts, resulting in an increased production of this factor (Kim et al. 1989). This suggests that growth arrested fibroblasts may also be able to modulate their own behaviour e.g., stimulation of a number of functions including growth factor production, migration, proliferation, ECM contraction and ECM synthesis. This hypothesis is supported by the fact that addition of exogenous TGFβ has been shown to overcome the inhibition of scarring, following exposure to MMC, in vivo (Khaw et al. 1994a). It is also interesting to note that some patients who receive antimetabolites during glaucoma filtration surgery, still exhibit scarring (The Fluorouracil Filtering Surgery Study Group, 1996). As such these findings may have significant implications to the healing process in vivo, in that the quantity and profile of growth factors produced following exposure to antimetabolites may influence the surgical outcome.

The profiles of production of growth regulatory molecules by control cultures and growth arrested fibroblasts seen in this study, is not an isolated one. The decreasing production of eicosanoids by control cultures of 3T3 fibroblasts as they reached
confluence, compared to an increased production of these molecules by MMC growth arrested 3T3 fibroblasts has been reported (Blacker et al. 1987). However, the reasons for these findings and the mechanisms of action of MMC upon this process, were not discussed. The reasons for production of growth factors by growth arrested cells in excess of control levels in this study, is currently unclear. One of the mechanisms of action of 5FU and MMC is the inhibition of RNA and protein synthesis (Chabner, 1982). However, single, five minute exposures to these agents did not appear to inhibit total RNA or protein synthesis. Exposure to high concentrations of 5FU (25mg/ml) and MMC (0.1mg/ml) significantly reduced TGFβ1 mRNA levels while resulting in significantly elevated levels of TGFβ1 protein, compared to controls. This suggests the possibility that high levels of antimetabolites resulted in the production of aberrant mRNA and subsequently non-functional TGFβ1 protein. Lower concentrations of 5FU and MMC did not appear to have this effect, with the production of TGFβ1 mRNA and protein appearing similar to controls.

Although both 5FU and MMC have been reported to cause breaks in DNA (Chabner, 1982), they did not cause cell death across the range of concentrations used in this study. Therefore, another possible mechanism of stimulating growth factor production following exposure to antimetabolites may involve a sublethal cellular injury/cellular stress response. Several reports in the literature have suggested that an increase in bFGF production by cells occurs as a result of exposure to various forms of stress including wounding, hypoxia and ionising radiation (Clarke and Feeback, 1996; Sakaki et al. 1995; Lee et al. 1995). Additionally, bFGF has been shown to be secreted from cells following sublethal injury (D’Amore, 1990). The role of bFGF in this stress response is unclear,
although it has been implicated in the protection of cells from hypoxic injury (Sakaki et al. 1995). A similar role for bFGF in this study is suggested by the upregulation of its production at the earliest time point studied (day 3), followed by a sustained decrease towards control levels. This phenomenon was observed at all concentrations of both agents tested, apart from exposure to 25mg/ml 5FU. The production of bFGF by these cells exposed to high concentrations of 5FU appeared to increase to a maximum at day 36 post exposure. If it is true that bFGF is produced as a response to sublethal injury, these results may suggest that the degree of injury/stress sustained by cells exposed to 25mg/ml 5FU is high. The degree of cellular injury or stress appeared to be related to the concentration of this agent used. It is possible that the intracellular metabolism of 5FU results in a prolonged presence of a derivative(s) that produce sustained injury / stress, or alternatively a single episode of damage could result in a prolonged response.

Like bFGF, TGFβ production was upregulated in all groups of growth arrested cells compared to control cultures, the effects appearing concentration dependent. It is possible that these increases in production of TGFβ, like bFGF, occurred as a result of sublethal cell injury or stress. However unlike bFGF, maximal production of TGFβ occurred at slightly later time points (days 7 to 36). Although the exact mechanisms underlying this process are unclear it may be that the initial increase in bFGF, with a resultant stimulation of the FGF receptor, leads to an upregulation of TGFβ production. Additionally, stimulation of fibroblasts with TGFβ has been shown to have an autoinductive effect on the production of this growth factor (Kim et al. 1989), an effect that has also been demonstrated on growth arrested fibroblasts at the mRNA level (Khaw et al. 1994a). It is possible that these mechanisms, either alone or in combination, could at
least contribute to the increases in TGFβ production. EGF was not detectable at either the mRNA or protein levels in all cultures of Tenon's capsule fibroblasts in this study. This lack of EGF production by these cells may not be surprising, as other workers have reported the lack of production of the EGF-related growth factor TGFα by ocular fibroblasts (Khaw et al. 1991a).

Although several hypotheses have been suggested above for the mechanisms underlying the effects of antimetabolite induced growth arrest on growth factor production, their validity is unknown. The production of other growth factors, such as members of the PDGF and IGF families, was not investigated in this study although these factors are known to influence fibroblast behaviour (Deuel and Huang, 1984; Gullberg et al. 1990; Narayanan and Page, 1983; Bird and Tyler, 1994; Khaw et al. 1994b). It should also be noted that the ELISA techniques used to quantitate growth factors in this study gave no indication of the biological activity of these factors produced by growth arrested cells. Potential further investigations of interest may include: the elucidation of the rate and exact nature of the cell's response to possible stress/injury via antimetabolites; the full range of growth factors produced and their biological activity. Such studies may lead to an increased understanding not only of the above mechanisms, but also cell-cell interactions.

The expression of growth factor receptors, both at the mRNA and protein levels, appeared to be reduced upon exposure to both 5FU and MMC. Although the exact mechanisms underlying this response were not determined in this study, it may be that exposure to these agents reduces receptor numbers by antimetabolic effects on mRNA and
subsequent protein production. Alternatively this reduction in receptor numbers may result as an overall cellular response to DNA damage following exposure to these agents, or may be due to a combination of DNA damage and antimetabolic effects. Interestingly, the expression of growth factor receptors in control cultures in this study appeared to be in the order of TGFβ type II receptor > bFGF receptor > EGF receptor. These finding may partially or fully explain the differential potencies of the growth factors TGFβ, bFGF and EGF on the stimulation of fibroblast functions observed by Khaw et al (Khaw et al. 1994b), who reported a decrease in potency in the order of TGFβ > bFGF > EGF for equimolar concentrations.

Although this study quantitated the expression of cell surface growth factor receptors, no indication of the biological activity of these receptors was obtained. It may therefore be of further interest to determine if the growth factor receptors expressed by growth arrested cells elicit intracellular effects, ultimately resulting in changes in cellular behaviour. Additionally, it is also interesting to speculate that a level of growth factor receptor expression may be required to elicit changes in cellular behaviour. Finally, similar studies on the range of other growth factor receptors including PDGF and IGF receptors, their numbers and biological activity, may provide further insight into the functional capabilities of growth arrested cells.

In summary, growth arrested cells appear to be capable of producing growth factors and expressing growth factor receptors. These findings may partly explain why growth arrested feeder layers are capable of supporting keratinocyte growth in vitro. Additionally, as both growth factors and growth factor receptors are involved in the regulation of cellular wound healing behaviour, the production of possibly biologically
active growth factors and receptors may also partly explain the scarring that in some instances occurs \textit{in vivo} following exposure to antimetabolites.

4.2.2 Extracellular matrix (ECM) molecules

Exposure to 5FU or MMC significantly reduced the production of collagen type I, collagen type III and fibronectin both at the mRNA and protein levels, throughout the 48 day assay period. The notable exception to the above effects was collagen type III mRNA production by cells exposed to high 5FU (25mg/ml). Whereas mRNA levels produced by these cells were significantly elevated compared to controls, this was not reflected at the protein level. These results suggest that both 5FU and MMC reduce the production of ECM molecules primarily via their antimetabolic effects on RNA and subsequent protein synthesis. The biological significance of these findings are currently unclear. It may be that although the levels of ECM molecules were reduced following growth arrest, compared to controls, their production may still be significant enough to support keratinocyte growth \textit{in vitro} or to contribute to a healing response \textit{in vivo}.

4.3 Growth Arrested Fibroblasts Exhibit Wound Healing Behaviour

4.3.1 Proliferation

The proliferation of human Tenon's capsule fibroblasts in this study was found to be inhibited in a concentration dependent manner by single, five minute exposures to 5FU. Whereas cells exposed to high concentrations (25mg/ml) were growth arrested for a prolonged period (36+ days) without cell death, those exposed to lower concentrations (0.25 and 2.5mg/ml) were growth arrested for 7-14 days after which proliferation started
again. High concentrations of MMC (0.1 mg/ml) induced growth arrest for a period of 36 days, the cells then beginning to proliferate. Following exposure to lower concentrations of MMC (0.001 and 0.01 mg/ml), cells did not appear fully growth arrested although their rate of proliferation was significantly reduced. These findings were similar to those reported from in vitro studies by Khaw et al. (1992b). This group also demonstrated the in vivo significance of these findings in that single exposures to antimetabolites increased the success of glaucoma filtration surgery, by reducing post-operative scarring via the reduction of fibroblast proliferation at the wound site. However, the exact mechanisms of how these single, five minute exposures to antimetabolites induce long term growth arrest are currently unclear. From the literature, it appears that both 5FU and MMC elicit several effects, any or all of which may result in growth arrest.

The primary antiproliferative mechanism of action of 5FU, and its derivatives produced via intracellular activation, is the inhibition of the enzyme thymidylate synthetase. The fact that derivatives of 5FU also elicit these effects is supported by a recent study by Panadero et al. (1995b), who demonstrated that 5FU was approximately 1000 times less potent an antiproliferative agent than one of its derivatives, 5-fluoro-2-deoxyuridine (F UdR). The biological consequence of thymidylate synthetase inhibition by these agents is a resulting thymidine deficiency. As thymidine is required for DNA synthesis, the result is a cessation of cell replication. In addition to resulting in a thymidine deficiency, exposure to 5FU has also been reported to elicit changes in the integrity of regulatory genes. These effects occur prior to the inhibition of cell proliferation, but subsequent to the inhibition of thymidylate synthetase (Panadero et al. 1995b). This group suggested that molecular alterations subsequent to thymidylate
synthetase inhibition may be responsible for the observed growth arrest. This hypothesis is supported by findings from studies by (Li et al. 1994) that c-myc expression (a nuclear transcription factor associated with cellular proliferation) was altered following exposure to FUdR. Recently Chu et al (1994) showed that thymidylate synthetase protein binds to c-myc mRNA and may therefore be involved in its regulation of expression and/or function.

Several studies have suggested that exposure to fluoropyrimidines including 5FU also results in DNA damage. This includes the production of 1 - 5 megabase fragments of DNA, in which the misrepair of uracil is thought to play a role (Sedwick et al. 1981). The generation of these breaks in cellular DNA have been suggested as determinants of the biological response to these agents, as well as being critical molecular lesions associated with cell death (Yoshioka et al. 1987; Hirota et al. 1989; Hutchinson, 1989; Blocher et al. 1989; Jeggo, 1990). The study by Panadero et al also showed that while 5FU affected the integrity of newly synthesised DNA, FUdR produced extensive single strand breaks in both mature and newly synthesised DNA. Although both 5FU and FUdR inhibit thymidylate synthetase, the higher potency of FUdR may result from its more extensive effects on DNA. The mechanisms of action of MMC include the cross-linking of DNA, which alters the DNA template and inhibits further DNA synthesis, free radical formation leading to cellular damage, and single strand DNA breakage (Chabner, 1982).

As highlighted in Section 1, DNA damage can have profound effects on a cell's ability to divide. This process of shutting down the machinery involved in DNA synthesis is thought to involve several mechanisms including: an upregulation of the 'genome
protector' p53; an increase in the cellular levels of p21 (resulting in an inhibition of the cyclin dependent kinases, which are normally required for progression through the cell cycle); and an increase in the expression of growth arrest associated genes (see Figure 1.8). As such, the cellular effects of both 5FU and MMC may be mediated via these molecules.

Whether one, a combination, or all of the above mechanisms account for the long term growth arrest seen following exposure to antimetabolites, is not known. However, what seems likely is that all of these molecular mechanisms play a role in the growth arrest process. This hypotheses is supported by findings from a number of studies which suggest that exposure to DNA damaging agents such as MMC results in the upregulation of p53 (Kastan et al, 1991; Zhan et al, 1993; Fritsche et al, 1993). Additionally, exposure to thymidylate synthetase inhibitors has been shown to suppress c-myc and upregulate p53, dephosphorylation of the retinoblastoma gene product (Rb) and induce DNA fragmentation (Arredondo et al. 1994; Panadero et al. 1995a), the effects being dependent on the concentration of agent used. These findings suggested that thymidylate synthetase protein may act upon binding to c-myc by regulating other genes such as p53, Rb and p21. Therefore a decrease in thymidylate synthetase, due to the effects of inhibitors such as 5FU, appear to cause a downregulation of c-myc expression, followed by a subsequent upregulation of growth arresting mechanisms. This hypothesis is further supported by findings showing that growth arrest, in a breast carcinoma cell line, was associated with a decrease in c-myc expression and an increase in p21 levels (Maas et al. 1995).
In summary, there are several possible mechanisms of action of growth arrest induced by single, five minute exposures to antimetabolites. The effects of 5FU may be due to the inhibition of thymidylate synthetase and/or direct DNA damage. The length of growth arrest appeared to be dependent upon the concentration of 5FU used. It is interesting to speculate that exposure to 5FU resulted in an upregulation of the above mechanisms blocking DNA synthesis. The length of growth arrest may therefore depend upon the concentration of the agent used and hence the degree of DNA damage requiring repair, or the degree of inhibition of c-myc expression. As regards MMC, it seems possible that the extent of DNA damage elicited by single, five minute exposures was also concentration dependent. The resulting degree of growth arrest, mediated by molecular mechanisms including p53 upregulation, may have reflected the degree of DNA damage sustained. Although the exact mechanisms involved in the process of growth arrest following exposure to antimetabolites are currently unclear, the above data suggests that further studies are required. Possible targets for investigation may include both the expression and interaction of the molecules highlighted above, including p53, retinoblastoma gene product and p21.

4.3.2 Migration

The migratory capacity of fibroblasts exposed to antimetabolites was reduced compared to controls. Recovery of cellular function during these experiments was similar to the results of proliferation, in that the migration of fibroblasts following exposure to 5FU appeared to consist of a lag-phase for 7-14 days prior to these cells exhibiting a
marked increase in migration. The effects of exposure to MMC had similar, but less marked effects than 5FU.

Other studies have reported conflicting data on the effects of growth arrest on cellular migration. Yamamoto et al (1990) reported that exposure to 5FU or MMC, although causing growth arrest, did not inhibit the ability of rabbit Tenon's capsule fibroblasts to migrate, using an agarose drop technique. Reports from Hogg et al (1995) suggested that growth arrest induced by exposure to sodium butyrate, reduced the ability of human trabecular meshwork cells to migrate. Unlike the Yamamoto study, both this current study and that performed by Hogg et al used migration assays based upon modified Boyden chambers, i.e., the ability of cells to migrate through pores in membranes. The conflicting data from the Yamamoto study may be explained either by species or migration assay differences. Additionally, this current study using single, five minute exposures to 5FU and MMC differed from the continuous exposure to these agents (Yamamoto et al. 1990) or continuous exposure to a different growth arresting agent; sodium butyrate (Hogg et al. 1995). As such, it is difficult to directly compare the data from these studies.

How cellular migration is inhibited by single, five minute exposures to 5FU and MMC is currently unclear. As highlighted in Section 1, the process of migration involves several co-ordinately acting cellular processes including growth factor receptors, integrins, the actin cytoskeleton and intracellular signalling cascades (see Figures 1.6 and 4.1). The results from this study showed that exposure to antimetabolites did not affect the settlement of cells onto the migration membranes. Presumably, this process is mediated by
integrins and so it seems unlikely that the antimetabolites were eliciting their effects via this mechanism. Actions upon the cytoskeleton also seem unlikely as the morphology of cells exposed to antimetabolites were similar to that of controls. A potential role for growth factor receptors in the anti-migratory effects of 5FU and MMC was suggested by this study, as a down-regulation of growth factor receptors following exposure to these agents was noted. The profiles of recovery of migratory ability by cells exposed to 5FU or MMC closely resembled those seen for proliferation. As such, it is possible that the mechanisms modulating the cellular responses for proliferation also affect migration. Therefore in a general context it may be that in response to DNA damage that complicated processes such as proliferation and migration, requiring the co-ordination of several intra- and extracellular mechanisms, are 'shut down' until the cell successfully repairs the damage sustained. However, further investigation is required to elucidate the exact mechanisms underlying the inhibition of this process. Potential targets for study may be: determination of the molecules, their roles and interactions, upregulated due to DNA damage; determination, quantitation and the roles of the regulatory molecules involved in this process, including growth factor receptors/integrins.

The fact that growth arrested cells are still able to exhibit long term migration may be significant to the healing process in vivo. It may be that these cells are eventually able to move into and around the wound site and elicit a scarring response, via other aspects of their healing behaviour. Such aspects may include the contraction of the ECM/wound, as this process is regarded as resulting from the tractional forces generated by migrating cells upon their substratum (Harris et al. 1981; Ehrlich and Rajaratnam, 1990).
4.3.3 ECM contraction

Results from the effects of single applications of 5FU or MMC on cell-mediated collagen contraction in this study, showed that these agents inhibited this process. However, a significant amount of collagen lattice contraction still occurred over the 7 day assay period, even at concentrations that induced complete growth arrest over the same time period in this and other studies (Khaw et al. 1992b). A number of agents have been shown to inhibit cell-mediated collagen contraction through mechanisms including: direct cytotoxicity (Heath et al. 1990; Heath and Hedlund, 1984); inhibition of protein synthesis (Adams and Priestley, 1988; Heath et al. 1986; Guidry and Grinnell, 1985) or protein glycosylation (Heath et al. 1990); inhibition of cytoskeletal function (Bell et al. 1979; Guidry and Grinnell, 1985); disruption of lattice structure (Guidry and Grinnell, 1987); inhibition of early cell-matrix interactions (Adams and Priestley, 1988; Zoutewelle and van Wijk, 1990); or increases in cyclic AMP levels (Ehrlich et al. 1983; Van Bockxmeer et al. 1984). However, the methods of treatment in these studies differed from the short, single exposures employed in this study.

Exposure to high concentrations of 5FU or MMC appeared to elicit cytotoxic effects (only in lattices containing nonactivated cells for 5FU) and affect the cytoskeleton, suggesting their anti-contractile effects may be due to these findings. However, at concentrations of these agents that did not significantly affect cell viability, lattice cellularity or the cytoskeleton, lattice contraction was still significantly inhibited. One of the major modes of action of 5FU and MMC is the inhibition of proliferation. As cells did not proliferate in this model of collagen contraction the mechanism(s) of inhibition appear uncertain. It is possible that 5FU and MMC may be eliciting their effects via antimetabolic
action upon RNA and subsequent protein synthesis (Tahery and Lee, 1989), as has been described for fluoropyrimidines (Heath et al. 1986).

Another issue addressed in this study was the dependence of antimetabolite effects on the state of cellular activation. Glaucoma patients receiving topical medications exhibit an increase in conjunctival cells numbers, scarring response and chance of surgical failure (Sherwood et al. 1989; Lavin et al. 1990), which appears to be associated with an increase in cellular activation (i.e., increased Ki67 expression) on histological sections (P. Khaw - personal communication). Although the absolute increase in cell number during healing in these patients is small, the state of activation of these cells may be a critical factor. Activated and nonactivated fibroblasts, seeded at the same densities, contract collagen lattices at different rates. Single exposures to 5FU or MMC inhibited lattice contraction in both activated and nonactivated cells, but the degree of inhibition was greater over the 7 day assay period in lattices populated with nonactivated cells than their activated counterparts. This is contrary to what is expected, because activated (proliferating) cells are usually more sensitive to antimetabolic / antiproliferative agents. It is possible that the nonactivated cells were compromised by their serum-free period, but there was no difference in trypan blue uptake indicating loss of cell integrity, and the cells still appeared healthy by phase contrast microscopy. This nonactivated state was shown to be reversible, in that nonactivated cells in control lattices were able to contract lattices fully under constant serum stimulation in the long term, albeit at a slightly slower rate.
The mechanisms underlying these observations are currently unclear. However, in the context of the current literature on this process and on antimetabolites, several possibilities are apparent. Guidry and Grinnell (Guidry and Grinnell, 1985) have hypothesised that cells contain a significant pool of factors necessary for cell spreading, lattice reorganisation, or both. It is interesting to speculate that endogenous cell factors required for the contraction process are present in activated cells in sufficient quantities for contraction to occur, before the inhibitory effects of 5FU or MMC on the synthesis and use of these factors manifest themselves. Nonactivated cells may not contain sufficient quantities of these factors and, as a result, the inhibitory effects of 5FU or MMC appear more pronounced. Additionally, another contributing factor for this apparent lag-phase before the inhibitory effects of 5FU take effect is that the metabolites of 5FU are more potent effectors of antimetabolic / antiproliferative actions than 5FU itself (see Section 4.3). The time taken for 5FU to be converted to these other molecules, which then elicit an anti-contractile effect, may be reflected in the ability of cells to partially contract the lattices. Interestingly, the dependence of the anti-contractile effects of antimetabolites on the state of cellular activation in the short term (over a 7 day assay period), was not found to be true for long term experiments (48 day assay period) suggesting that partial recovery of function occurs. However, contraction of the ECM is an 'early' healing event and so the state of cellular activation may influence the healing response in vivo. Finally, the actual degree of cellular activation in vitro may not be exactly the same as the degree of cellular activation in vivo. Nonetheless, that single applications of antimetabolites have different effects depending upon the state of activation of the target cell may have important clinical implications. These include the possible use of preoperative applications of these agents prior to glaucoma filtration surgery.
One other observation of interest from this study was that cells exposed to MMC appeared to become non-viable at the lattice-medium interface, prior to those located mid-lattice. This suggests that the penetrative quality of the extracellular matrix may also be a critical factor in the efficacy of these agents. In the clinical situation, the conjunctiva is more dense and varies in thickness compared with collagen lattices, and the antimetabolites are only applied from one side of the conjunctiva. These findings may partly explain the variation in response to these treatments by patients who are otherwise similar.

In summary, following single, five minute exposures to antimetabolites cells appeared to still be able to contract collagen lattices before the inhibitory effects of exposure to antimetabolites manifested themselves. This suggests that these cells may still contribute to the overall healing response \textit{in vivo}. The fact that cells still contracted collagen to a certain degree following exposure to antimetabolites prior to the manifestation of inhibitory effects is in contrast to the other aspects of cellular behaviour studied, i.e., proliferation and migration. The differences in these effects may be due to the fact that the contraction study was performed with cells in ECM while the others were in monolayer culture systems, suggesting the response of cells to antimetabolites may be influenced by the ECM. It may therefore be of interest for future investigations to develop pseudo-\textit{in vivo} models to study these aspects of cellular behaviour, in order to mimic more closely the effects of these agents \textit{in vivo}.
4.4 Role of Matrix Metalloproteinases (MMPs) in Extracellular Matrix

Contraction

As highlighted in section 1.3.1.2, the process of fibroblast-mediated collagen contraction is not only a crucial component of the healing process, but also other fundamental biological processes including tissue morphogenesis and embryonic development (Grinnell, 1994; Stopak and Harris, 1982; Lewis, 1984; Brenner et al. 1989). Although this contractile process has been shown to involve several key components including: the ability of cells to attach to their matrix; protein synthesis; an intact actin cytoskeleton; and the generation of tractional forces by migrating cells within their matrix (Schiro et al. 1991; Klein et al. 1991; Guidry and Grinnell, 1985; Bell et al. 1979; Harris et al. 1981; Ehrlich and Rajaratnam, 1990), the exact mechanisms of this process are currently unclear. For fibroblast migration into the wound site \textit{in vivo} to occur, these cells must have the capacity to penetrate their surrounding ECM with the ECM essentially 'opening up' to provide space for this penetration (McCarthy et al. 1988). Presumably as these cells migrate into the wound site \textit{in vivo}, the tractional forces exerted upon the ECM by their movement causes matrix contraction.

It was noted during this study, as have other researchers, that cells populating and contracting collagen lattices undergo a series of morphological changes; all requiring cellular penetration of the surrounding matrix. Results from this study on the effects of antimetabolites on collagen contraction indicated the presence of a series of cell-associated holes / tunnels within contracted collagen matrices. Although some of these holes may have been artefacts, these findings suggested the possibility of an enzymic activity, namely the MMPs, being a component of this contractile process.
4.4.1 MMPs are produced during, and are required for, intra-matrix cell-mediated collagen contraction

Previous reports in the literature have suggested possible mechanical mechanisms for cell mediated collagen contraction (Gabbiani et al. 1972; Ehrlich and Rajaratnam, 1990) and that MMPs were not critical to the contractile process (Mauch et al. 1989; Lambert, 1992; Seltzer et al. 1994). However, these studies used MMP inhibitors of low potency to provide their evidence. The results from this study showed that upon culture within and during the contraction of collagen lattices, both MMP mRNA and protein were markedly upregulated. These findings are similar to those of several other studies investigating the behaviour of cells interacting with ECM molecules (Mauch et al. 1989; Seltzer et al. 1994; Huhtala et al. 1995; Riikonen et al. 1995). However, none of these studies tried to directly link MMPs with contraction of the ECM.

Using three different potent, broad spectrum MMP inhibitors (Galardin™-MPI, BB-94, CellTech), at comparable concentrations to those used in other biological systems (Gearing et al. 1994; Gijbels et al. 1994; McGeehan et al. 1994), this study indicated that MMP activity was required for collagen contraction by cells seeded within the matrix. In addition to the model of collagen contraction used in this study, with fibroblasts entrapped within a three dimensional matrix (similar to the in vivo situation), other studies have employed a model involving the seeding of cells onto the matrix surface (Schor, 1980; Guidry and Grinnell, 1985). Using a similar model in this study, it was found that during culture upon and contraction of this collagen matrix that MMP activity was produced. The gelatin zymogram profile of the MMPs produced was the same as that seen for cells seeded within collagen lattices. However, quite surprisingly exposure to the MMP
inhibitors Galardin™-MPI, BB-94 and CellTech did not elicit any anti-contractile effects when cells were seeded upon the collagen lattices.

These results suggested that contact with a collagen matrix either in two or three dimensions (cells seeded upon or within lattices respectively) upregulated the expression of MMP activity. Additionally, the fact that inhibition of MMP activity inhibits collagen contraction by cells within but not upon the matrix, suggests that there may be two different mechanisms underlying the process of collagen contraction in these different models; one consisting of MMP activity requirement for cellular movement inside collagen (cells in lattices), and one consisting of cellular movement on top of collagen independent of MMP activity.

Following these experiments, non-specific anti-contractile effects of these MMP inhibitors could not be excluded. However, subsequent investigations demonstrated that the MMP inhibitors did not adversely affect lattice cell viability or number, the actin cytoskeleton or the attachment of cells to their surrounding matrix, all of which have been demonstrated as crucial components of contraction (Bell et al. 1979; Buttle and Ehrlich, 1983; Guidry and Grinnell, 1985; Schiro et al. 1991; Klein et al. 1991). The lack of effect of MMP inhibitors on these cellular functions required for contraction is further corroborated by the data in this study upon the effects of MMP inhibitors on collagen contraction by cells seeded upon the lattice surface. Presumably, if the MMP inhibitors were affecting these cellular functions then an inhibition of contraction by cells seeded upon the surface of lattices would have been observed; this was not the case.
In addition to the production of MMPs (1, 2 and 3) during contraction, TIMP 1 was also produced. This result is not particularly surprising as TIMP 1 is usually secreted complexed with MMPs (Birkedal-Hansen, 1995). As highlighted in Section 1, it is the balance of the ratios between MMPs and TIMPs that regulate overall MMP activity. Although the concentrations of TIMP 1 increased during collagen contraction it was nowhere near as much as the MMPs, suggesting an overall net increase in MMP activity during collagen contraction. Only MMPs 1, 2 and 3 were produced during collagen contraction, MMP 9 was not. The production of MMP 1 (collagenase) and MMP 2 (72kD gelatinase) may be explained by the fact that the major substrates of these two enzymes are collagen type I (for collagenase) and gelatin (for 72kD gelatinase), both of which would be major constituents off the collagen lattices during contraction. However, the major substrates for MMP 3 (stromelysin) are proteoglycans. So why was MMP 3 produced during collagen contraction? One possible explanation for this is that MMP 1 and MMP 3 are often co-ordinately expressed, as the promoter regions of these genes have several common regulatory sequences (Ries and Petrides, 1995). Alternatively, it may be possible that the commercially available type I collagen used in this study contained 'contaminating' proteoglycan material; a possibility which requires further investigation.

In this current study, results suggested that MMP activity was a crucial component of fibroblast mediated collagen contraction regardless of the tissue site or species the fibroblasts were derived from. Video time lapse data suggested that cells exposed to MMP inhibitors were unable to spread, penetrate and move through their surrounding matrix (unlike controls), a process which was associated with an inhibition of contraction. Similar findings relating the requirement of MMP activity for the penetration and movement of a
number of cell types through ECM have been reported. These include smooth muscle cells (Bendeck et al. 1994; Pauly et al. 1994); T cells (Leppert et al. 1995a; Leppert et al. 1995b; Romantic and Madri, 1994); PC12 (rat pheochromocytoma) cell growth cones (Nordstrom et al. 1995); cytotrophoblasts (Librach et al. 1991); glioma cells (Nakagawa et al. 1995); biliary cells (Terada et al. 1995); and retinal growth cones (Sheffield et al. 1994). In this current study, although cells exposed to MMP inhibitors were able to produce small cytoplasmic processes, they were unable to undergo the further changes in morphology and movement associated with collagen contraction in this model.

In summary, the exact mechanisms underlying the movement of cells through ECM and its subsequent contraction were not determined in this study. However, what was apparent was that cell spreading, penetration and movement of cells through their surrounding matrix was facilitated by MMPs, and this ultimately resulted in contraction of the collagen lattices.

4.4.2 Possible molecular mechanisms of MMP actions, and the regulation of MMP activity, in collagen contraction

Two possible speculative molecular mechanisms, either alone or in combination, could account for the observations outlined above. Firstly, MMPs may be involved in the direct localised degradation of the matrix immediately surrounding the cell. This would allow cell spreading and penetration of the matrix, with the subsequent formation of holes or tunnels (in conjunction with tractional remodelling of the matrix) which then facilitate the migration of cells through the matrix, and their subsequent contraction of the matrix.
(this hypothesis is illustrated schematically in Figure 4.2). The second hypothesis differs slightly from the first in that instead of degrading the surrounding matrix, the MMPs may 'open up' the matrix by cleaving some sort of collagen inter-fibrillar bond(s). This may allow the penetration of cell processes, which leads to the subsequent exertion of tractional forces upon the matrix, as in the first hypothesis. This may in turn lead to the formation of holes or tunnels for further cell spreading and/or migration to occur, and ultimately contraction of this matrix (see Figure 4.3). Both of these hypotheses suggest that the matrix surrounding cells would be modified by enzymic activity, and that collagen bundling via exertion of tractional forces would occur. This may lead to the formation of a series of intra-matrix holes or tunnels; a phenomenon that was observed in this study.

The regulation of MMP activity during cell mediated collagen contraction is currently unclear. From this and other studies, cellular contact with ECM components either in two (cells on) or three (cells in) dimensions appear to result in the upregulation of MMP activity (Hunt et al. 1993; Saarialho et al. 1993; Woodley et al. 1986; Tremble et al. 1994; Mauch et al. 1989; Azzam and Thompson, 1992; Seltzer et al. 1994). However, as shown in this study, it is only when cells are seeded within collagen matrices that MMP activity is required for contraction. The initial extracellular signalling molecules involved in MMP induction following ECM contact appear to be the integrins (Riikonen et al. 1995; Langholz et al. 1995; Mauch et al. 1989; Seltzer et al. 1994; Huhtala et al. 1995), these molecules also being reported as essential to the contraction of collagen matrices (Schiro et al. 1991; Klein et al. 1991). Although the process of collagen contraction has also been shown to involve intracellular signalling cascades with molecules such as protein kinase C (Guidry, 1993), again the exact intracellular mechanisms involved are unclear.
Several candidate molecules could be involved in the process of extracellular signal transduction to the nucleus, with resultant changes in gene expression, protein production and ultimately cellular behaviour. It would be of interest to investigate the roles of such molecules in the control of cellular migration and contraction of collagen, in order to further understand the biological mechanisms underlying these crucial processes. As highlighted in Section 1 (Figure 1.6), several of these candidate molecules include members of the Rho family of small GTPases (e.g., Rac and Rho), PI3-kinase and cytoskeletal associated proteins (e.g., profilin Theriot and Mitchison, 1993; Sohn and Goldschmidt-Clermont, 1994; Danielsen et al. 1988). The potential roles and possible targets for further investigation of these molecules are highlighted in Figure 4.4.
Figure 4.2: Potential role of MMPs in fibroblast spreading, migration and matrix contraction, within collagen lattices: Degradation of matrix

- De/attachment to matrix
- Localised secretion of MMPs
- Matrix degradation and modification
- Extension of cytoplasmic processes

- Continued secretion / action of MMPs
- Degradation of matrix
- De/attachment to matrix
- Further cell spreading / elongation
- Modification of matrix

- Continued secretion / action of MMPs
- Further matrix degradation
- De/attachment to matrix
- Cell elongation, bipolar morphology
- Migration of cells
- Generation of tractional forces
- Matrix modification and reorganisation
Figure 4.3: Potential role of MMPs in fibroblast spreading, migration and matrix contraction, within collagen lattices: 'Loosening' of matrix

- De/attachment to matrix
- Localised secretion of MMPs
- 'Loosening up' of matrix
- Extension of cytoplasmic processes

- Continued secretion / action of MMPs
- Further loosening of matrix
- De/attachment to matrix
- Continued cell spreading and elongation
- Modification of matrix

- Secretion / action of MMPs
- Continued loosening and modification of matrix
- De/attachment to matrix
- Further cell elongation, bipolar morphology
- Migration of cells
- Generation of tractional forces
- Collagen bundling and matrix reorganisation
Figure 4.4: Schematic representation of the processes involved in cell movement through collagen and collagen contraction

Cell morphology during lattice contraction and production of MMPs/TIMP

Potential involvement of signal transduction molecules
Possible Targets for Investigation

Spherical immediately post seeding

MMP Levels
Medium MMP mRNA
Low MMP Protein

Penetration of cells into surrounding matrix

MMP Levels
High MMP mRNA
Medium MMP Protein

Cell elongation and bipolar morphology.

Increased cellular migration

Cell surface actin stress fibres disappear

Reorganisation of Matrix

MMP Levels
Low MMP mRNA
High MMP Protein

Compacted Matrix
Contraction Ceases

Response to external stimuli

Production of lamellipodia and cytoplasmic processes

Rac
PI3-kinase

Attachment to ECM via integrins

Integrins
Rho

Initial presence of cell surface actin stress fibres

Profalin Rho
4.4.3 Antimetabolites, MMPs and collagen contraction

It was demonstrated in this study that exposure to antimetabolites, at certain concentrations, inhibited collagen contraction without causing cell death. This fact taken in conjunction with the requirement of MMP activity for contraction, suggested that 5FU and MMC may inhibit contraction by inhibiting MMP production.

Quantitation of the levels of MMPs, produced during collagen contraction by ELISA, suggested that exposure to high concentrations of 5FU (25mg/ml) reduced MMP levels, while lower concentrations either had a stimulatory or no effect. Gelatin zymogram analysis of MMP biological activity suggested a 5FU concentration-dependent decrease in MMP activity, 0.25mg/ml 5FU not significantly affecting levels compared to controls. Similar results were obtained with MMC. These results suggest that exposure to antimetabolites elicit a concentration-dependent reduction in MMP production during collagen contraction. This reduction was only apparent upon analysis of actual biological activity, suggesting that some non-functional proteins may be produced following exposure to antimetabolites.

In summary, MMPs appear to be produced during the contraction of collagen regardless of whether the cells are seeded within or on top of the matrix. However, this MMP activity is only required for contraction by cells seeded within the matrix. The contraction of collagen is regarded as resulting from the generation of tractional forces by cells migrating upon their substratum. It was shown in this study that cells within collagen matrix required MMP activity to spread, penetrate and finally move through the collagen matrix. It appeared that it was this process of cell spreading, penetration and migration of cells through the matrix that resulted in the generation of intra-matrix tractional forces and
subsequently the contraction of the matrix. This requirement of MMP activity for collagen contraction appeared to be true regardless of the tissue site or species the fibroblasts were derived from. Additionally, the anti-contractile effects of single, five minute exposures to the antimetabolites 5FU and MMC appeared to be due, at least in part, to a reduction of MMP activity.

Finally, not only does contractile scarring play a crucial role in the pathogenesis or failure of treatment of many visually disabling or blinding conditions including cataracts, glaucoma, trachoma, burns, proliferative vitreoretinopathy (following retinal detachment) and age related macular degeneration, it is also a cause of clinical morbidity throughout the body. Examples include the disfiguring, disabling and painful scarring seen following thermal, chemical and radiation burns; scarring following injury or surgery, e.g., internal adhesions and internal blockage; and scarring due to disease, e.g., scleroderma and cirrhosis. The movement of cells through the extracellular matrix and the subsequent contraction of collagen containing tissues are crucial components of this scarring response. As such, the novel findings in this study may have important therapeutic implications in that specific, non-toxic, locally applied agents which inhibit MMP activity may be useful in controlling scarring and scar tissue contraction throughout the body. Furthermore, MMP inhibitors may be useful investigative tools in teasing out the basic mechanisms underlying complex processes involving cellular movement within the ECM and collagen contraction such as tissue repair, embryonic development and tissue morphogenesis.
4.4 Conclusions

The results in this thesis have shown that human Tenon's capsule fibroblasts are capable of exhibiting varying degrees of both molecular and cellular aspects of wound healing behaviour, following single, five minute exposures to the antimetabolites 5FU and MMC. These included: the expression of growth factors and receptors for TGFβ and bFGF, but only the EGF receptor, at both mRNA and protein levels; the expression of collagen types I and III, and fibronectin at the mRNA and protein levels. Exposure to antimetabolites also induced growth arrest and reduced the ability of cells to migrate, with cells eventually fully or partially recovering these functions. Cells were also able to partially contract collagen matrices prior to the inhibition of this process by 5FU and MMC, the effects appearing to be due to the degree of cellular activation. Additionally, MMPs were identified as a crucial and possibly ubiquitous component of fibroblast-mediated collagen contraction and movement within the ECM.

These experiments have suggested that cells may be capable of exhibiting wound healing behaviour following exposure to antimetabolites, that are widely used to reduce scarring in vivo. As such, these findings may partly explain why, following these treatments, scarring is still seen in some instances. The demonstration that MMPs appear to be involved in cell movement through ECM and its subsequent contraction, may have implications for new anti-scarring strategies. Finally, the experiments performed in this thesis have posed many more questions for the future understanding of growth arrest and fibroblast behaviour.
References


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Gospodarowicz, D., Neufeld, G., and Schweigerer, L. Molecular and biological characterization of fibroblast growth factor, an angiogenic factor which also controls the proliferation and differentiation of mesoderm and neuroectoderm derived cells. Cell. Differentiation. 19:1-17, 1986c.


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Appendix  (equipment and material suppliers)

Agar Scientific, Stanstead, Essex, U.K.
Amersham, Little Chalfont, Bucks, U.K.
BDH/Merck, Lutterworth, Leics, U.K.
Biogenesis, Yoemans Park, Bournemouth, U.K.
Bio-Rad, Hemel-Hempstead, Herts, U.K.
Boehringer-Mannheim, Lewes, East Sussex, U.K.
Chemicon, Harrow, U.K.
Costar, High Wycombe, Bucks, U.K.
Coulter Electronics, Luton, Beds, U.K.
Cruachem, Glasgow, U.K.
Dakopatts (Dako), High Wycombe, Bucks, U.K.
Data Cell, Maidenhead, U.K.
David Bull Laboratories, Warwick, U.K.
EuroGenetics, Teddington, Middlesex, U.K.
Fisons, Loughborough, Leics, U.K.
Fuji Chemicals, Toyama, Japan.
Gibco Life Technologies, Paisley, Scotland, U.K.
Hybaid, Teddington, Middlesex, U.K.
ICN Flow, High Wycombe, Bucks, U.K.
Jeol, Welwyn Garden City, Herts, U.K.
Jandel Scientific, Corte Madre, California, USA.
Kurta, Phoenix, Arizona, USA.
Kyowa, Essex, UK.
Lab Impex, Teddington, Middlesex, U.K.

Leica, Lowhill, Milton Keynes, U.K.

Marathon, London, U.K.

NeuroProbe, Cabin John, MD, U.S.A.

Novex (see R and D Systems)

Olympus, London, U.K.

Perkin-Elmer, Warrington, Chesire, U.K.

Pharmacia, St Albans, Herts, U.K.

Polaron (Fisons), Uckfield, East Sussex, U.K

Phillip Harris, London, U.K.

Promega, Chilworth Research Centre, Southampton, U.K.

Qiagen, Dorking, Surrey, U.K.

R and D Systems, Abingdon, Oxon, U.K.

Sartorius, Epsom, Surrey, U.K.

SPSS Inc., Chicago, U.S.A.

Unipath, Basingstoke, Herts, U.K.

Whatman, Maidstone, Kent, U.K.

Wilj International Ltd., Ashford, Kent, U.K.

Zeiss, Welwyn Garden City, Herts, U.K.