THE EFFECT OF MECHANICAL LOAD ON DERMAL FIBROBLAST COLLAGEN DEPOSITION AND ORGANISATION

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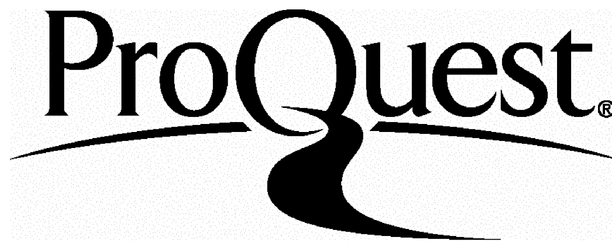

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ABSTRACT

During dermal wound healing, resident cells are constantly exposed to mechanical forces from surrounding tissue movement. It is hypothesised that these forces contribute to increased human dermal fibroblast collagen deposition and alignment, and that this external mechanical signal is transduced to the cell nucleus via the cell surface receptors integrins. To address this hypothesis, two *in vitro* mechanical loading systems have been employed: a 2-dimensional system to load dermal fibroblasts in monolayer culture and a 3-dimensional system to load cells within a collagen gel. The effect of mechanical load in the presence of serum or growth factors on procollagen synthesis and deposition was determined in each system. It was found that mechanical load alone in either system had no effect on procollagen synthesis. However, in the presence of 10% fetal calf serum or TGFß, both loading regimens lead to a highly significant stimulation of procollagen synthesis. Levels of the enzyme procollagen c-proteinase, critical in the formation of insoluble collagen fibrils, were also increased in response to load, whereas the procollagen c-proteinase enhancer protein was not. In the 3-dimensional system there was also alignment of collagen fibrils in response to load, and this was shown to be dependent on the load-induced increase in collagen synthesis. The involvement of integrins in load-induced procollagen synthesis in the 2-dimensional system was investigated. Integrin $\alpha1\beta1$ was shown to be increased at the cell surface in loaded cultures, and important in basal procollagen synthesis. $\alpha2\beta1$ was transiently increased at the cell surface by mechanical load, and was specifically involved in mediating load-induced collagen synthesis and deposition. $\alpha5\beta1$ was not involved in collagen synthesis is this system. These data demonstrate that mechanical load and growth factors act synergistically to enhance human dermal fibroblast collagen synthesis, deposition and re-organisation, and that these phenomena are in part regulated by specific integrins. The elucidation of these mechanisms has furthered the understanding of fibroblast responses to mechanical load, and may assist in the development of novel therapeutic strategies to treat pathologies involving enhanced dermal scarring.

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LIST OF ABBREVIATIONS

BMP-1 .... bone morphogenic protein-1

BSA .... bovine serum albumin

CO_2 .... carbon dioxide

DMEM .... dulbeccos modified eagles medium

DNA .... deoxyribonucleic acid

ECM .... extracellular matrix

EDTA .... ethylenediaminetetracetic acid

FAK .... focal adhesion kinase

FCS .... fetal calf serum

FGF .... fibroblast growth factor

HCl .... hydrochloric acid

HDF .... human dermal fibroblast

HPLC .... high pressure liquid chromatography

Hz .... Hertz

MMP .... matrix metalloproteinase

MOPS .... morpholoino-propanesulphonic acid

NaOH .... sodium hydroxide

NBDCl .... chloro-nitrobenz-oxa-diazole

NF-1 .... nuclear factor-1

NO .... nitric oxide

OHpro .... hydroxyproline

PBS .... phosphate buffered saline

PCP .... procollagen c-proteinase

PCPE .... procollagen c-proteinase enhancer

PDGF... platelet derived growth factor

PKC ....protein kinase C

RGD .... Arg-Gly-Asp

RNA ....ribonucleic acid

rRNA .... ribosomal ribonucleic acid

SMC ....smooth muscle cell

SSC... sodium citrate/sodium chloride solution

TBS... tris-buffered saline

TGFß .... transforming growth factor ß

TIMP .... tissue inhibitors of metalloproteinases

SDS-PAGE............sodium dodecylsulphate-polyacrylamide gel electrophoresis

CHAPTER ONE

General introduction

1. GENERAL INTRODUCTION

Tissue repair, which includes the healing of skin, organs and bone is an enormously complex process, and thus presents challenges with regard to understanding and modification in a therapeutic capacity. Wound healing and restoration in the skin is vital to homeostasis and survival of the individual as it forms a protective barrier. Therefore, understanding the molecular processes is essential to open the way to therapeutic intervention in aberrant healing and scarring, and further, fibrosis and tumour development.

The mechanical forces in the tissue surrounding a healing dermal wound are constantly changing due to movement of skin and muscles. It is known from clinical studies that these mechanical forces play a role in altering the final scar morphology. A reduction in tension can reduce the size of the final scar, however, the biochemical basis for this phenomena is not yet understood.

This thesis reports the investigation of the effects of mechanical forces on skin fibroblasts, with a view to extrapolating findings to wound healing and scar formation *in vivo*. In particular, the effects of load transduction on collagen deposition, and the specific integrin receptors responsible for load transduction are investigated. In this

introduction, the effects of mechanical loading both *in vivo* and *in vitro* and the properties of collagen and integrins and will be discussed.

1.1 WOUND HEALING AND TISSUE REPAIR

Self-repair is defined as the capacity to recognise damage to normal tissue and facilitate repair through physiological adaptations to maintain or restore normal function and in some cases, appearance. A wound is a pathological condition where damage to a tissue may cause separation or destruction, and is often associated with loss of function. Subsequent to damage, healing proceeds over a range of time courses largely dependent on the severity of the wound. Cutaneous wounds can be classified into three major categories:

Partial thickness wounds:

> Wound damage to the epidermis and upper dermis only. Re-epithelialisation occurs from both the wound edges and remains of epidermal appendages. Scarring occurs in some cases.

Full-thickness wounds:

> The epidermis, dermis and appendages removed, meaning re-epithelialisation only occurs from the edge of the wound, a process associated with extensive contraction. Scarring occurs in the majority of cases.

Complex wounds:

Deep wounds, which affect tendons, bones, ligaments and organs. Healing takes much longer and gives poorer cosmetic and functional results. Scar tissue is always formed.

The process of wound healing although complex is extremely ordered, and proceeds through four successive but overlapping phases; Homeostasis, inflammation, proliferation and remodelling.

1.1.1 Homeostasis

All events involved in wound repair are initiated at the moment of injury when damage to the vasculature causes peripheral blood to enter the wound site. Contact with collagen causes platelets to aggregate and degranulate leading to the release of a-granules, which contain many cytokines including PDGF and TGFß, as well as proteins involved in the coagulation cascade. Formation of the fibrin clot through a sequential proteolysis and polymerisation cascade leads to haemostasis. This clot acts as a scaffold (or provisional matrix) for inflammatory cells, and later neovasculature and fibroblasts (Grinnell, 1981; Plow, 1985; Gailit, 1994).

1.1.2 Inflammation

Following formation of the clot, vasoconstriction and vascular permeability allow the influx of inflammatory cells into the wound site. This triggers a phase of acute inflammation, leading to localised increase in neutrophils and macrophages. The recruitment of these cells is mediated by a number of factors which include fibrin degradation products (Ray et al, 1995), fibrinopeptides (Senior et al, 1986) and growth factors released from platelets (Kingsnorth et al, 1986; Slavin, 1992). Monocytes and macrophages clear contaminating bacteria (via phagocytosis) from the injured tissue site, but also release a number of proteases which participate in the removal of damaged tissue from the wound matrix. Macrophages continue to accumulate after neutrophil influx has ceased and release a number of growth and chemotactic factors necessary for the initiation of granulation tissue formation (Dipietro et al, 1995; Schaffer et al, 1996). Macrophages are essential to the wound repair process, mainly due to their secretion of a range of growth factors including TGFß, IL-1 and TNF-α (Grotendorst, 1992 for review). In addition to these cells, lymphocytes, mast cells and other plasma cells are known to be involved in wound repair, although their roles are currently still being characterised.

1.1.3 Proliferation

This phase involves the events associated with the repopulation of the wound, predominantly by fibroblasts, keratinocytes and endothelial cells. Dermal healing is mediated by the fibroblasts, and these cells convert the fibrin clot into the final scar tissue, and are therefore the key to successful cutaneous repair.

Fibroblast migration into the clot begins around three days following the initial insult. After migration into the wound, proliferation begins, which is stimulated by a number of fibroblast growth and chemotactic factors, which originate mainly from platelets and macrophages, but also from the fibroblasts themselves. During the synthesis of new tissue, the fibroblast alters from a migratory phenotype to a collagen producing phenotype (Gabbiani et al, 1972). Finally, fibroblasts assume a contractile phenotype, when cells have characteristics of normal mesenchymal cells, such as the expression of α–smooth muscle actin, vimentin and desmin, and these are often referred to as myofibroblasts (Skalli et al, 1989; Desmouliere et al, 1992; Gabbiani et al, 1992; Estez et al, 1994).

The loose extracellular matrix (ECM) initially produced by the fibroblasts is primarily composed of fibronectin and hyaluronic acid and is termed granulation tissue (Clark et al, 1993; Gailit et al, 1994). Other ECM components, along with tenascin (Kanno et al, 1994) may act as a scaffold for initial fibroblast migration through the wound space and regulate fibroblast biology (Brown et al, 1993; Greiling et al, 1997). Subsequently, over the following seven days, ECM composition alters, resulting in type III collagen becoming the major component with the fibronectin matrix possibly acting as a scaffold for fibrillogenesis (McDonald et al, 1983; Greiling et al, 1997). Organisation of the nascent collagen is achieved partly through wound contraction. However, the degree of organisation never reaches that observed in normal skin. Scar tissue appears more striated microscopically, and has a maximum of 70% the tensile strength of normal dermis (Levenson et al, 1965).

1.1.4 Remodelling

The composition and structure of the continuously changing granulation tissue depends on both the time elapsed since injury, and the distance from the wound margin. Maturation of the matrix results in a reduction of the fibronectin and hyaluronic acid content within the granulation tissue and an increase in the amounts of collagen bundles and proteoglycans such as chondroitin sulphate and dermatan sulphate. At this stage however, the wound has only gained around 20% of its' final tensile strength. Thereafter, the rate at which the wound gains tensile strength is much slower, and the rate of collagen synthesis in the scar does not return to normal until 6-12 months. Metalloproteinases, including the collagenases, gelatinases and stromelysins, as well as the plasminogen activator/plasmin systems are all thought to play an important role in this remodelling process (Romer *et al*, 1994; West *et al*, 1997). The tissue inhibitors of metalloproteinases (TIMPs) (Stricklin *et al*, 1993; Tamia *et al*, 1995; Ashcroft *et al* 1997) are also thought to be vital in controlling the rate of matrix degradation by MMPs during both *in vivo* normal and pathological wound repair.

The process of wound contraction is closely associated with remodelling events, and it is thought that contraction is the main method through which the wound closes (Clark 1993; Gailit et al, 1994). In humans contraction is often problematic, and can cause cosmetic and functional impairment including loss of joint motion in very severe instances. Research into the mechanisms of wound contraction have focused on the wound fibroblast (Grinnell et al, 1994) and the heterogeneity of fibroblast populations (Erwin et al, 1994; Desmouliere 1995; Phipps et al, 1997). The rapid accumulation of

fibrillar collagen and the remodelling of the wound *via* myofibroblast-driven matrix contraction is usually complete around 20-25 days after initial wound formation. The maximum strength of a final scar tissue is only 70% as strong as intact skin (Levenson *et al*, 1965), thereby making the site more susceptible to further damage.

1.2 MECHANICAL LOAD

1.2.1 Effects of mechanical load on tissue function

Mechanical load is known to have wide reaching effects on many of the different tissues and organs throughout the body. Most tissues are constantly exposed to mechanical forces through normal physiological processes such as breathing and movement. Many tissues have successfully adapted to this extraneous tension and in some cases stretch is actually necessary for the correct developmental processes to take place. However, in situations following tissue damage, the effects of exogenous tension are less well understood, and in these cases load may indeed contribute to a more severe phenotype.

Tension is as driving force for muscle protein synthesis and growth. Innervated, normal muscle, will not hypertrophy in the absence of mechanical load, yet load alone can trigger hypertrophy (Goldspink, 1978). Hypertrophy occurs in all muscles in

response to load, and leads to increased protein synthesis and a genetic switch leading to an altered, more efficient muscle phenotype (Laurent and Millward, 1980; Parker et al, 1989). Similarly, in the uterus, an increased artificial load causes increased protein synthesis (Douglas et al, 1988). This is then reversed when the uterus undergoes shrinkage, for instance post-partum. Both strain and compression are stimulus for growth, remodelling and matrix synthesis of bone and tendon cells (Harter et al, 1988; Jones and Bingmann, 1991). Conversely, bone mass is lost if the tissue is immobilised or subjected to zero or low gravity suggesting load is important in maintenance of normal bone structure.

The lungs and cardiovascular system have also been well studied in the context of mechanical forces. The lungs are constantly subjected to mechanical forces during both development and normal breathing. However, load exerts its most profound effects following pneumonectomy, whereby the remaining lung undergoes re-growth to replace the previous cells and matrix architecture (Riley et al, 1990). The mechanical load exerted upon this remaining portion of lung is hypothesised to be the main stimulus for this growth (Brody et al, 1978). The cardiovascular system is another example of a group of tissues existing in a highly mechanical environment. The tissues in this system are able to respond to increased, variable mechanical loading patterns. Increased mechanical load in both the heart and the vasculature stimulates remodelling in the form of increased ECM protein expression (Villarreal and Dillmann, 1992; Bishop et al, 1990) and muscle protein (Baird et al, 1987). The responses to load are cell specific, and can therefore cause hypertrophy of cardiac and vascular muscle, as well as increased ECM proteins and replication in mesenchymal

cells (Leslie et al, 1991; Keeley et al, 1993; Bishop et al, 1990). These examples all highlight the powerful consequences of stretch in different *in vivo* systems.

The dermis has not been well studied in terms of responses to mechanical loading in vivo. Clinical studies suggest that mechanical forces in the dermis following injury or during wound healing can influence scar formation (Sommerlad and Creasey, 1979). However, the biochemical reasons for this are very poorly understood. Clearly, as the examples in other systems highlight, stretch can stimulate growth and new protein synthesis in many systems and therefore may likewise play a very important role in similar processes in the skin.

1.2.2 In vitro mechanical loading systems

In order to test the role of mechanical load in cell activation, many different *in vitro* mechanical load systems have been developed. Research groups in the field designed the early loading systems often using very basic devices to apply direct loads to muscles. Many of the more developed and sophisticated systems are now commercially available (Banes et al, 1985; Terracio et al, 1988). Most of these devices generate a uni- or bi-axial strain field on the cell monolayers with the cells cultured on a variety of different flexible membranes. Direct mechanical stimulation using weights, or positive/negative air pressure applied beneath the membrane may be used to deform the cells.

The first cell straining devices were developed around 20 years ago, and initially only exerted a simple uniaxial strain on smooth muscle cells on purified elastin membrane (Leung et al, 1976). Bi-directional systems were then designed to exert forces on myotubes (Leung et al, 1977). Since then a large number of biaxial systems have been well characterised in the literature, including the Flexercell Strain Unit used in this thesis (Glibert et al, 1994). These systems can either be used to exert cyclical or static loads upon the cells, depending upon the hypothesis being tested. The increasing development of these systems also allows the use of different ECM-coated plates for analysis of matrix dependent stretch responses.

An alternative form of *in vitro* loading device is based around a 3-dimensional model using a matrix gel containing cells. Many studies investigating both contraction processes and the effects of endogenous cellular mechanical tension utilise cells embedded in collagen gels (Bell et al, 1979). In these systems, cells are allowed to contract collagen gels, or gels are "tethered" to the culture plate to allow the generation of endogenous tension. The only 3-dimensional culture system which applies endogenous mechanical load to collagen gels is the tensioning culture fore monitor (T-CFM) also used in this thesis (M. Eastwood, PhD Thesis, University of Westminster, 1996). This device applies a uniaxial cyclical load to cells embedded in a collagen gel. The increasing availability of loading systems has stimulated a rapidly increasing body of literature describing complex biochemical and physiological processes involved in stretch-mediated responses.

1.2.3 Mechanical load and activation of cells in vitro.

Mechanical forces regulate function *in vitro* in cells from many different organs and systems. Load has been shown to activate cells derived from tendons, ligaments and the lung (Bishop et al, 1994, Gilbert et al, 1994) and increase total procollagen synthesis in cardiovascular fibroblasts (Butt and Bishop, 1997). Mechanical forces also stimulate replication and growth factor production in smooth muscle and endothelial cells (Wilson et al, 1993; Sumpio et al, 1987). Activation of ion channels and release/generation of second messengers occurs within seconds of the application of loading in many cell types (For review, see Sachs et al, 1997). Within minutes or hours of stretching the cell, changes in gene transcription or altered cell morphology is seen. Finally, long term responses such as changes in protein synthesis, cell replication and differentiation, and protein degradation occur over a course of many hours or days.

Re-orientation is also a common response to mechanical load and has been studied by a number of different groups. This phenomenon has been widely reported in endothelial cells (Ives et al, 1986, Dartsch and Betz, 1989), cardiac fibroblasts (Bishop et al, 1993), smooth muscle cells (Dartsch et al, 1986) and myocytes (Terracio et al, 1988). Alignment has been studied principally in monolayer cultures, and in these systems, cells exhibit alignment either perpendicular or parallel to the direction of strain. This response to stretch may represent the normal alignment of cells necessary to maintain structural integrity of the tissue in development and repair *in vivo*. A small

number of papers have also reported cell alignment within 3-dimensional collagen gels, in which the collagen fibrils have been aligned using magnetic forces (Dickinson *et al*, 1994). However, the dependence of the orientation on cell function in response to load has not yet been well determined. Clearly, the effects of mechanical load on dermal fibroblast and collagen orientation are of great interest with respect to the alignment of collagen fibrils seen in normal scar formation (Sommerlad and Creasy, 1977). The theory of load-induced collagen fibril alignment is one which will be addressed within the 3-dimensional collagen gel loading system in this thesis.

Fibroblasts are subjected to both active and passive tensions - contraction of the cell or forces exerted from the surrounding ECM. Fibroblasts grown in retracted collagen lattices, a recognised *in vitro* model of wound contraction, demonstrate a decrease in collagen synthesis, both at transcriptional and translational levels, a decreased collagenase activity and changes in cell morphology (Eastwood et al, 1996; Mauch et al, 1988; Nakagawa et al, 1989). The combined evidence from *in vivo* and *in vitro* studies highlights the diversity of effects mechanical load can exert upon cell activity and matrix deposition. As mentioned previously, dermal fibroblasts are not well characterised in any *in vivo* or *in vitro* models of exogenous mechanical forces. However, as mechanical load clearly plays an important role in mesenchymal cell activation in the *in vitro* systems tested, it is important that these concepts are investigated in skin fibroblasts to assist in the understanding of dermal responses to stretch. Elucidation of the mechanisms, and mediators, of the effects of load may allow the development of novel therapies designed to treat conditions involving collagen deposition and scar formation.

1.2.4 Clinical management of scars: influence of mechanical forces

Clinical observations have shown that the less mechanical load that is exogenously applied to a scar *in vivo,* the smaller and finer the final scar is (Meyer and McGrouther, 1991). This would suggest that external load is playing a part in the final orientation of the matrix in the scar tissue, and thereby directly affecting the remodelling and reorientation processes involved in the wound healing response. It is hypothesised here that the interaction of load and specific growth factors controls the final cell orientation, collagen production rate, the ratio of collagen types and the final orientation of the cells and collagen fibres.

The risk of poor healing resulting in unsightly scars can currently be reduced in a number of ways. Effective planning of surgical incisions, for instance along the natural relaxed skin tension lines (line of least stretch), reduces the number of directions of loading upon the wound thereby decreasing the amount of gaping and consequently overall scar size. Skin is also being subjected to altering degrees and directions of stretch during movement. This dynamic loading does affect scar maturation, but currently there are very few ways available to prevent this form of mechanical stimulation, other than strapping of wound margins or splintage where possible. Incised wounds are generally sutured, relatively loosely to prevent ischaemia leading to overall breakdown and stretching of the wound edges.

Stretched scars develop when the forces acting perpendicular to the wound edges exceed the tensile strength of the immature collagen fibres. This can be caused by closure of a wound under excessive tension, movement of underlying muscles and joints, abnormalities in fibroblast activity or collagen formation, or subsequent growth (Pape, 1993). Following suture removal, there is little to oppose the tendency of the wound to gape, and the wound can either break down or the intact dermal layer becomes thin and attenuated. Again, there is little, other than strapping or a non-absorbable sub-cutaneous suture, to provide prolonged support for the wound. Once a stretched scar has occurred, only surgical revision can alter it (Elliot and Mahaffey, 1989). It is currently not known whether the tension development is more important in scar formation or if load is the key modulator. However, it has already been established *in vitro* that mechanical loading of cardiac fibroblasts induces procollagen synthesis (Butt and Bishop, 1997), and this observation is likely to be relevant to the dermal wound healing setting. It has also been established that the orientation and physical positioning of a wound *in vivo* can dramatically influence the shape of the wound following closure. This has been attributed to the different mechanical tensions apparent within different parts of the body, which exert different strains on the wound environment. (Sommerlad and Creasy, 1978. Kengesu et al, 1993)

1.3 THE COLLAGENS

Given that the hypothesis is that mechanical load is influential in determining the final size of a scar via their effects on collagen deposition and fibril organisation, it is important to consider at what stages in the process of synthesis, deposition and degradation forces may act.

1.3.1 Structure

The interstitial collagens, consisting of three polypeptide-chains composed of large helical domains are synthesised as procollagens (pro-chains) which undergo processing to α-chains and subsequently assemble into collagen fibrils and fibres. Each α-chain consists of a central left-handed helical region containing the characteristic repeating triple unit of amino acids, Gly-X-Y (where Gly is glycine, X is frequently proline and Y the imino acid 4-hydroxyproline), as well as short non-helical regions at the N- and C-terminal ends. The Gly-X-Y sequence is essential for the folding of the molecule into a right-handed triple helix, stabilised by inter-chain hydrogen bonds. Glycines, having no side chains are capable of being positioned in the centre of the triple helix (Traub & Piez, 1971). As a result, peptide bonds are buried within the interior of the molecule, this structure rendering the triple-helical region highly resistant to proteolysis.

The precursor molecules for the interstitial collagens contain three separate domains; the central, triple-helical portion, described above; an N-procollagen peptide domain consisting of a terminal globular region of 77-86 amino acids, a small triple helical region of about 40 amino acids and a short, 2-8 amino acid non-helical domain linking the pro-peptide to the helical chain (the cleavage site for the removal of the N-propeptide) and a globular C-terminal region, stabilized by inter-chain disulphide bonds. These propeptides, comprising one third of the total mass, are removed by N- and C-terminal peptidases, leaving short non-helical regions at both ends of the molecule, containing sites for cross-linking to other collagen molecules.

1.3.2 The collagen superfamily

Thirty-three distinct collagen chains exist, interacting to form the different collagen types. Collagen types may exist as homotrimers composed of three identical α–chains (collagen III: $[\alpha_1(III]_3)$ or as heterodimers consisting of two (collagen I: $[\alpha_1(I)]_2\ \alpha_2(I)$) or three (collagen VI: $\alpha_1(VI)\ \alpha_2(VI)\ \alpha_3(VI)$) different α-chain subunits. To date, at least 21 members of the collagen superfamily have been identified, each functionally and structurally distinct and coded for by at least 30 different genes, widely distributed throughout the genome (van der Rest & Garrone, 1991).

Collagens type I and III are encoded for by separately transcribed genes; $\alpha_1(I)$ and $\alpha_2(I)$, and $\alpha_1(III)$ respectively. These genes are known to be upregulated *in vitro* in fibroblasts by a multitude of different factors, including TGFß, thrombin, ascorbate

and insulin (Geesin et al, 1988; Raghow et al, 1987; Dabbagh et al, 1998; Goldstein et al, 1989). The translated product, termed pre-procollagen is proteolytically cleaved to form procollagen. The procollagen α-chain then undergoes a series of post-translational modifications including hydroxylation of specific proline and lysine residues in the Y position of the recurring Gly-X-Y triplet to from 4-hydroxyproline and hydroxylysine. Procollagen molecules are eventually transported from the rough endoplasmic reticulum (RER) to the golgi where they are packaged into secretory vesicles and transported from the cell (Kivirikoo et al, 1989).

1.3.3 Procollagen synthesis

Transcription

Procollagen genes encoding for the pro-α_1 (I) and pro-α_2 (I) chains consist of about 18 and 38 kilobases (kb), respectively. Both genes contain about 50 introns, which are generally much shorter in the pro- α_1(I) than the pro- α_2(I) gene. The intervening exons consist of predominantly 54 or 108 base pairs (bp), a feature common to all of the so-called fibrillar collagen genes.

Procollagen DNA is transcribed to pre-mRNA in the nucleus. Pre-mRNA is spliced to form functional mRNAs (4-6 kb) before translation on membrane bound polysomes in the RER. The translated product, termed preprocollagen α-chain, contains an N-terminal hydrophobic signal peptide, which is thought to facilitate movement across the RER. This peptide is proteolytically cleaved, either during translation or shortly

after polypeptide synthesis (Nimni & Harkness, 1967). The resulting procollagen α-chain undergoes a series of co- and posttranslational modifications.

Regulation of procollagen gene transcription

The regulation of procollagen gene transcription is accomplished via gene regulatory proteins called *trans*-acting nuclear factors interacting with regulatory DNA sequences, which consist of short DNA stretches of defined sequence called *cis*-acting elements. The regulatory DNA sequences have been identified for several procollagen genes. They are usually located in the 5' end of the gene, upstream of the start site of transcription, they include the gene promoter, a region recognised by RNA polymerase II, although sequences located downstream of the transcription start codon in the first intron of a number of procollagen genes have also been identified. These include α_1(I), α_2(I), α_1(II), α_1(III) and α_1(IV).

Procollagen gene promoters typically contain a TATA box, necessary for the orientation of RNA polymerase II (Ritzenthaler *et al*, 1991). Several promoters have also been shown to contain CCAAT (or CAT) binding regions. The factors binding to this canonical sequence have been purified, cloned and sequenced. The binding of one such factor, CCAAT binding factor (CBF) has been demonstrated to be required for transcriptional activation of both the α_1(I) and α_2(I) procollagen genes (Maity *et al*, 1988, Karsenty *et al*, 1988, Karsenty *et al*, 1990). The regulation of the α_1(III) procollagen gene is similar to the type I procollagen genes in that in contains a TATA box and CCAAT binding regions. In contrast, the α_1(II) procollagen promoter

contains a TATA but no CCAAT box, while α_1(IV) and α_2(IV) procollagen genes have a CCAAT, but no TATA box.

Promoters of the collagen genes have been found to contain several other *trans*-acting nuclear factors, including activated gene regulatory protein (AP-1) sites, which bind the heterodimer composed of the activated forms of *fos* and *jun* proteins. AP-2, specificity protein-1 (SP-1) and nuclear factor-1 (NF-1) sequences have also been identified in some procollagen genes (Rossi *et al*, 1988, Karsenty *et al*, 1988). Procollagen genes also contain upstream negative regulatory elements which bind inhibitory regulatory factors.

Regulatory AP-1 and SP-1 DNA sequences located downstream of the transcriptional start site have been demonstrated in the first intron of many of the procollagen genes and may enhance or inhibit procollagen gene transcription (Liska *et al*, 1990, Bornstein *et al*, 1987). DNA methylation may also serve to regulate procollagen gene transcription, possibly as a negative regulatory control mechanism (Guenette *et al*, 1992).

Posttranslational modifications

Intracellular modifications of the newly synthesised polypeptide chains results in the formation of procollagen molecules containing an array of hydroxyproline and hydroxylysine residues. The principal modifications are the hydroxylation of prolyl and lysyl residues, brought about by the actions of three hydroxylases and two

glycosyl transferases. Most of these events occur as co-translational modifications while the nascent polypeptide chains are still growing on the ribosomes, but the reactions are continued as posttranslational modifications until triple helix formation of the pro α-chains occurs, which prevents any further processing.

Prolyl 4-hydroxylase catalyses the hydroxylation of proline during polypeptide elongation and proceeds until virtually all Y-positioned prolines are hydroxylated. Some proline residues in the X-position are also hydroxylated by prolyl 3-hydroxylase (Kivirikko *et al*, 1989). Hydroxyproline, unique to the collagens and a few other proteins (Campa *et al*, 1990), is essential for the stability of the triple helix. Under-hydroxylated procollagen cannot form triple helices and is therefore susceptible to degradation (Steinmann *et al*, 1981). Lysyl hydroxylase converts lysine to hyroxylysine (about seven residues per α–chain). The reaction mechanisms of all three enzymes are similar and each requires as cosubstrates Fe^{2+}, 2-oxoglutarate, molecular O_2 and ascorbate.

The galactosyl and glucosyl tranferases, hydroxylysyl galactosyl transferase and galactosyl hydroxylysyl glucosyl transferase respectively, transfer carbohydrate moieties to the hydroxyl groups of hydroxylysines in the helical regions (Kivirikko & Myllyla, 1984). The extent of this glycosylation is very variable, both between collagen types and within the same collagen in different tissues and at different ages. The biological role of the collagen-specific carbohydrate units remains unclear, although they may have some role in fibril organisation. A final modification

involves the transfer of mannose-rich oligosaccharides onto asparagine-linked carbohydrate units in the C-propeptides of fibrillar collagens and some domains in nonfibrillar collagens (Olsen *et al*, 1977).

Triple helix formation in the fibrillar collagens occurs through a nucleolation centre in the C-propeptide as a result of the alignment of three procollagen α-chains in a way that juxtaposes appropriate cysteine residues in the C-terminal propeptides (Engel & Prockop, 1991). This results in the formation of intra- and interchain disulphide bonds between opposing lysine and hydroxylysine residues. Triple helix formation proceeds in zipper-like fashion from the C-terminus to the N-terminal domain. The precise mechanism by which non-fibrillar collagens- lacking homologous C-domains common to the fibrillar collagens- are assembled is not known, but some of these collagens possess large non-triple helical domains, which may be functionally equivalent to fibrillar C-propeptide domains (Olsen, 1991).

Procollagen processing

The procollagen molecules then move from the endoplasmic reticulum to the golgi apparatus where they are packaged into small vesicles, form small aggregates and are carried to the cellular membrane (Kivirikko *et al*, 1989). During or immediately after secretion, the N- and C-propeptides of fibrillar collagens are enzymatically cleaved by procollagen N- and C-proteinase, respectively. Because cultured cells secrete predominantly procollagen into the culture medium, it is generally assumed that these

proteinases act extracellularly. However, whether this is the case *in vivo* has not been confirmed.

Subsequent cleavage of the propepetides leads to spontaneous assembly into fibrils by a number of enzymes at the cell plasma membrane (Birk *et al*, 1986). Included in these are the enzymes responsible for procollagen processing, a critical event in the deposition of new matrix. The C-terminal procollagen c-proteinase (PCP) has in particular been implicated, along with the specific PCP enhancer protein PCPE, as a rate-limiting enzyme in pathologies involving excessive collagen deposition, such as fibrosis and scarring (Ogata *et al*, 1997). These enzymes are likely to be regulated by many of the same factors regulating collagen synthesis, and recent work by Lee and colleagues suggest this is certainly true in the case of TGF-ß and ascorbic acid (Lee *et al*, 1997). Manipulation of these enzymes has already provided a more specific tool for the *in vitro* control of "fibrosis". The regulation and involvement of these enzymes in the "fibrotic" responses to mechanical load is examined in great detail in Chapter 4.

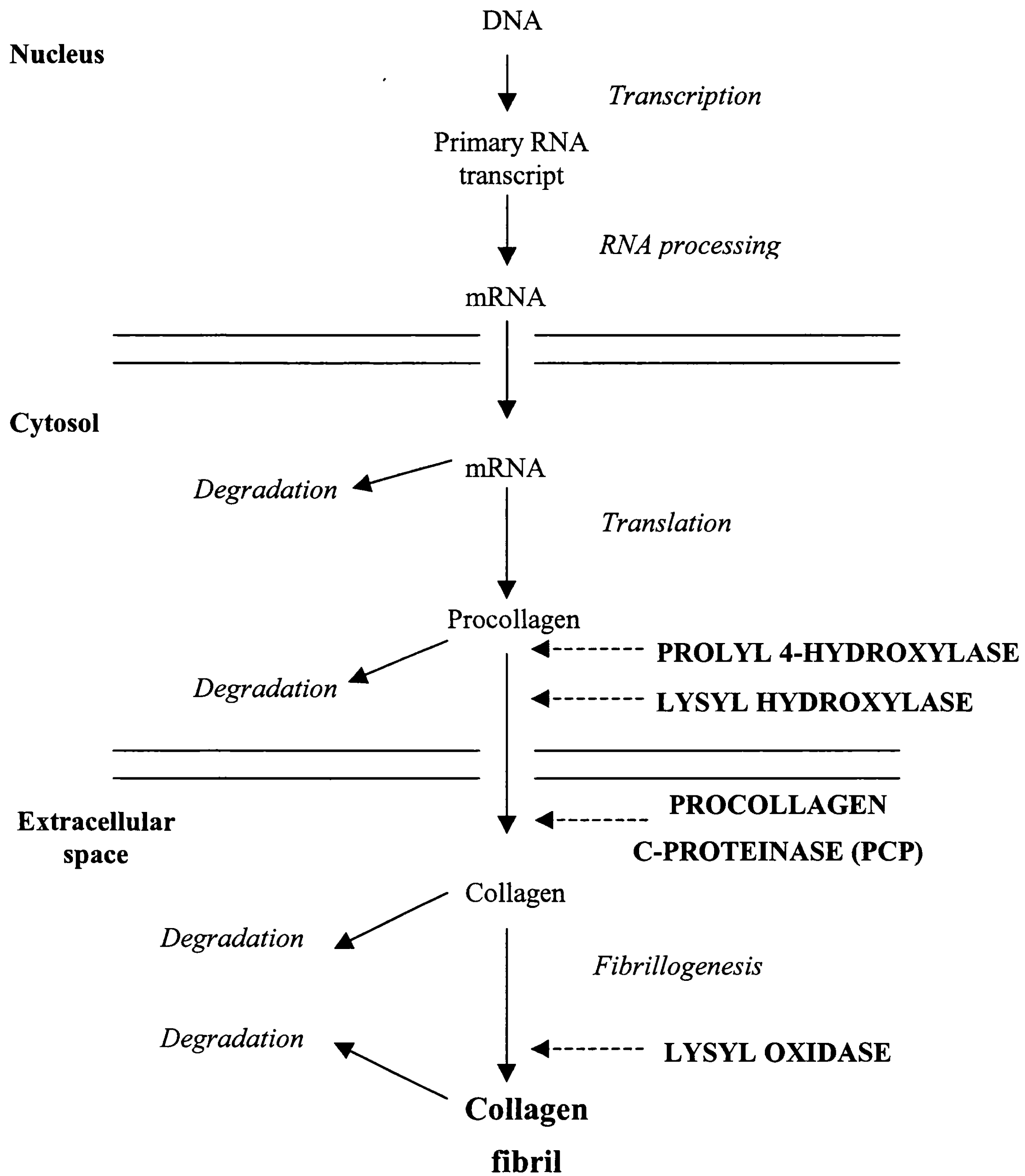
Fibril formation

Fibre formation appears to be a self-assembly process. The molecules align precisely in quarter-stagger arrays, first forming microfibrillar units, which are then packed together to form larger fibres (Birk & Trelstad, 1986). The fibres are stabilised by the formation of inter-molecular covalent cross-links catalysed by lysyl oxidase, which readily binds to molecules arranged in fibres (Yamauchi & Mechanic, 1988). Lysyl

and hydroxylysyl residues, particularly in the non-helical regions, undergo oxidative deamination to form allysine and hydroxyallysine.

Inter-molecular cross-links then form through generation of a Schiff base between the aldehyde-containing precursor and the side chains of a lysyl or hydroxylysyl residue in the helical region of an adjacent molecule. The nature of these cross-links is thought to change with age, from reducible cross-links described above to a stable non-reducible form, which occurs through the formation of polyvalent cross-links. There is some evidence that cross-links may form between type I and III collagen, supporting the immunohistochemical evidence that type I and III molecules appear to be present in the same fibril (Henkel & Glanville, 1982).

Fig. 1.1: Schematic diagram of procollagen synthesis and processing



1.3.4 Procollagen Degradation

Two principal pathways exist by which collagen may be degraded; an intracellular pathway that rapidly degrades procollagen molecules and an extracellular pathway involving the degradation of fibrillar collagen.

Intracellular degradation

Intracellular degradation is thought to occur mainly in lysosomes, although a small amount has also been shown to be non-lysosomal (Bienkowski, 1984). The regulation of intracellular degradation is poorly understood, but several non-specific proteases with other functions in the cell may be involved. The functions of intracellular degradation may be two-fold; to prevent secretion of defective molecules and secondly, to provide a level of regulation of collagen output in response to extracellular stimuli. Evidence in support of the former hypothesis comes from studies in which proline analogues are incorporated into the collagen chain, preventing them from forming a stable triple helix. These unstable collagen chains are rapidly degraded (Berg *et al*, 1980). Fibroblasts under-hydroxylate collagen during the log phase of growth, which is associated with the degradation of three times the proportion of newly synthesised collagen compared with cells which are confluent (Tolstoshev *et al*, 1981). The role of this degradative pathway as a regulator of the extent of collagen deposition has been demonstrated in studies of fibrotic disorders where enhanced collagen deposition is associated with a rise in the level of collagen degraded rapidly (Laurent & McAnulty, 1983).

Extracellular degradation

The extracellular degradation of collagens occurs largely as a result of the activity of matrix metalloproteases. Collagenolysis by collagenase may be regulated at several sites (Krane, 1985) including: (a) biosynthesis and secretion of the latent enzyme pro-collagenase predominantly by fibroblasts; (b) activation of the latent enzyme by proteases; (c) interaction of the active enzyme with the collagen substrate and (d) modulation of the effects of the active enzyme by proteinase inhibitors, such as tissue inhibitor of metalloproteinase (TIMP) and α_2-macroglobulin. Collagenase cleaves the collagen molecules, in fibres, into a large fragment, TC_A (75 % of the molecules length) and a smaller TC_B fragment. The cleavage site is closer to the C-terminal and is specific for the peptide bond between glycine and isoleucine. Once the collagen molecule has been cleaved, the triple helix unwinds and is degraded further by nonspecific proteases.

This degradative pathway is thought to play a role in the remodelling of the collagen matrix during rapid tissue growth, since this process involves the breakdown of existing cross-linked collagen fibres. The remodelling process is dependent upon both collagen synthesis and catabolism, the latter being controlled by a variety of collagenase enzymes. These enzymes, or matrix metalloproteinases (MMP's) are specific for particular types of collagens, and currently three MMP's with collagenase activity have been characterised (Grant *et al,* 1987; Hibbs *et al*, 1987; Saus *et al*, 1988). The activity of MMP's is controlled by tissue inhibitors of metalloproteinases (TIMP's) which are tightly regulated during wound repair (Brenner *et al*, 1989). This modulation may be controlled by cytokines such as PDGF, TGF-ß, and IL-1, and the

ECM itself may play an important role in the regulation of remodelling (Circolo *et al*, 1991; Sporn and Roberts, 1992).

1.4 GROWTH FACTORS IN TISSUE REPAIR

As mentioned above, tissue repair consists of a highly structured sequence of events, each stage being controlled by a number of mediators derived from inflammatory, resident and blood cells. Some of the key mediators in the wound healing process are detailed below, focussing on those studied throughout the course of this thesis.

1.4.1 Platelet Derived Growth Factor

A host of cytokines and polypeptide growth factors are released in the initial stages of tissue repair, mainly by the platelets and white blood cells. Platelet derived growth factor (PDGF) is one of them, and is a potent mitogen and chemoattractant for fibroblasts amongst other cell types (Seppa *et al*, 1982). It also upregulates collagen gene expression (Butt *et al*, 1995), fibronectin expression (Blatti *et al*, 1988) and collagenase activity (Bauer *et al*, 1985), all processes essential during early stages of wound healing. There are three dimers in the PDGF family, -AA, -AB and -BB, encoded for by 2 separate genes (Ross *et al*, 1990). PDGF isoforms are stored in platelets and also produced by macrophages, smooth muscle cells, vascular endothelial cells and fibroblasts. Besides the wide number of effects on cells, PDGF

can also induce expression of TGFß in fibroblasts, which may be a way of potentiating indirectly its role throughout the course of healing (Pierce *et al,* 1989). Recombinant PDGF-AB and -BB have been shown to accelerate healing in skin wounds (Pierce *et al,* 1988) and very recently have been used in clinical trials as therapy for chronic leg ulcers in humans (Falanga *et al*, 1998). In these situations, PDGF increases the number of fibroblasts invading into the wound site which enhances granulation tissue formation and collagen production (Robson *et al,* 1992).

1.4.2 Transforming growth factor-ß

TGFß is a critical mediator in tissue repair, always found at sites of dermal injury and therefore the role of this growth factor in collagen deposition is studied extensively throughout the course of this thesis. There are 5 isoforms of TGFß (Massague *et al,* 1992; Roberts and Sporn, 1993) only 3 of which are expressed by mammals (1-3). All mammalian isoforms of TGFß are capable of stimulating both fibronectin and collagen synthesis in fibroblasts (Roberts *et al*, 1990). TGFß1 is secreted by most mammalian cells, usually in the latent (non-active) form. The other 2 isoforms are not found in platelets but have high sequence homology to TGFß1 and appear to be functionally similar.

Recently, TGFß has been demonstrated in a number of studies to accelerate wound healing (see review by O'Kane and Ferguson, 1997) and significantly increases the breaking strength of incisional wounds in rats (McGee *et al,* 1989). This is mainly attributable to a marked increase in infiltration of inflammatory cells and fibroblasts,

and therefore increased collagen deposition at the wound site. This translates into increased scar size due to an increase in matrix production and indeed increased levels of TGFß itself (Quaglino *et al*, 1990). However, fibrosis is decreased in areas around the wound margins when anti-TGFß1 and anti-TGFß3 antibodies are used (Shah *et al,* 1996). More focussed studies have since been carried out investigating the anti-scarring potential of TGFß1-3. It was found that neutralisation of both TGFß1 and 2 is required at the time of wounding to reduce the amplification cascade of TGFß1 at the wound site and thereby decrease scarring (Shah et al, 1992; Shah *et al,* 1994).

1.4.3 Blood-related factors

The serine protease thrombin is involved in tissue repair as well as being involved in blood clotting; it has been shown to be important in the regulation of fibroblast procollagen production and proliferation, potentially acting via protease activated receptors (Dabbagh *et al,* 1998, Chambers *et al,* 1998 and Gray *et al,* 1995). Fibrinogen and fibronectin are the other blood-derived soluble proteins, which are thought to play a role in regulating wound healing. These proteins bind both ECM and cell-surface proteins and thereby provide a scaffold for cell proliferation and movement. The soluble breakdown products are also mitogens and chemoattractants for fibroblasts (Gray *et al,* 1993).

1.4.4 Other factors

There are a number of other growth factors and cytokines that play roles in the various processes of tissue repair. Fibroblast growth factor (FGF) is a family of factors

produced by activated macrophages. These are very potent mitogens for endothelial cells, and therefore are likely to be vital in angiogenesis by directing cell migration and proliferation and plasminogen activator synthesis (Montesano *et al,* 1986). FGF2 (bFGF) has been shown *in vivo* to accelerate granulation tissue formation by increasing fibroblast proliferation and collagen accumulation.

Epidermal growth factors (EGF) are another large family of molecules containing a subset of factors, which have an EGF-like domain within then, such as transforming growth factor-α (TGFα). Various studies have demonstrated EGF-induced enhancement in the rate of epithelial cell regeneration and in collagen deposition and tensile strength of full thickness wounds (Mathers *et al*, 1989). Topical application of EGF has also been used clinically to increase epidermal regeneration and resultant tensile strength of wounds and chronic leg ulcers (Brown *et al*, 1986; Brown *et al*, 1988; Falanga *et al*, 1992). In vitro studies have also confirmed a direct effect of EGF proteins on fibroblasts, keratinocytes, smooth muscle cells and epithelial cells (Schreiber *et al*, 1986; Laato *et al*, 1987).

Insulin-like growth factor (IGF) is a single peptide growth-promoting hormone found in plasma, which is very important in skeletal muscle growth and protein metabolism. IGF-1 is expressed by fibroblasts, smooth muscle cells and endothelial cells, and has been shown to be an active mitogen and promoter of procollagen synthesis (Gillery *et al*, 1992). This factor is known as a "progressive" one because alone, application of IGF will not stimulate replication, but replication is seen following pre-incubation

with PDGF (Stiles *et al,* 1979). IGF is present within healing wounds, and has been implicated in the proliferation of smooth muscle cells during cardiac hypertrophy, a disease characterised by collagen deposition and increased mechanical tension (Wolfe *et al,* 1993).

1.5 THE INTEGRINS

1.5.1 Integrin function

Integrins are the primary cell surface receptors for the extracellular matrix. They are expressed on all vertebrate cells, and most cells express more than one integrin. Rather than being merely ECM anchors, integrins are involved in a range of signalling, mechanotransducing and cytoskeletal modulating functions. Integrins transduce a number of signals that alter growth regulatory pathways (for reviews see Schwartz *et al,* 1995; Dedhar *et al*, 1996; Howe *et al*, 1998). These signals include the activation of the tyrosine kinases focal adhesion kinase (FAK), pp60src and c-Abl; serine threonine kinases such as mitogen activated protein kinase (MAPK) and extracellular regulated kinase (ERK). Intracellular effectors JNK and protein kinase C (PKC) are also integrin-regulated, as well as intracellular signalling ions calcium and sodium, the small GTPases of the Rho family, the lipid mediators such as phosphoinositides, diacyl glycerol (DAG), calcium signalling molecules and arachidonic acid metabolites. This wide range of functions demonstrates the

fundamental role integrins play in governing cell phenotype, often in response to a changing extracellular environment.

Integrins are responsible for communicating information to the cell about the immediate external environment, the modulation of that matrix/substratum through contraction and reorganisation and influencing the phenotype and functions of other cells, as well as migration, adhesion and programmed cell death. To allow this range of functions, the cellular molecules that associate with the cytoplasmic domains of the integrins must participate in a wide range of signalling and cytoskeletal mechanisms. There are considerable overlaps between the key downstream events, which participate in the cytoskeletal and biosynthetic/mitogenic responses.

The integrins are a family of heterodimeric proteins, which consist of one α and one β subunit. Integrins are functionally expressed at the cell surface and are responsible for binding to the ECM and other cells by binding to other integrins or other cell membrane adhesion molecules such as the intercellular adhesion molecules (ICAM)s, and in doing so form a conduit between the extracellular and the cellular cytoskeleton, to which they are attached. Integrin α subunits vary in size between 120 and 180 kDa, and each non-covalently associates with a slightly smaller β subunit. Many integrins are expressed by a wide variety of cells, and the majority of cells express a range of integrin heterodimers. So far only 22 integrins have been described out of the potential diversity which could exist. This is largely due to the fact that most α subunits associate with a single β subunit, and integrins are therefore sometimes classified

based on their β subunit. Integrins function at the simplest biochemical level, i.e. substrate specificity has been determined by adhesion assays, the use of function blocking antibodies and affinity chromatography. Such studies have revealed that most integrins bind more than one substrate (see Table 1.1).

Integrins show affinity for different ligands in a variety of cell types (reviewed in Meredith *et al*, 1997). The integrin recognition sites in the various ligands have been well defined. This began with the elucidation of the Arg-Gly-Asp (RGD) motif present in fibronectin, described initially by Rhouslahti and co-workers (Pierschbacher *et al*, 1984; Rhouslahti *et al*, 1987) and is now thought of as the "classical" integrin ligand. It is present in a variety of different proteins and is recognised by a large subset of integrins. Indeed, rather than β subunit classification families, recognition sequence is increasingly being used to classify integrins into groups (Hynes *et al,* 1992). Further recognition sequences have been reported in many ECM molecules, for example QAGDV and a variable motif termed LDV with the consensus sequence L/I-D/E-V/S/T-P/S (Humphries *et al,* 1996).

Integrins subunit structure is characterised by the possession of a large cytosolic glycoprotein domain, a single hydrophobic transmembrane segment and in most cases a short cytoplasmic tail. Each subunit is thought to form tight, compact domains due to extensive disulphide bridges, which render intact integrins resistant to proteolysis. Dimerisation is mediated through the extracellular domains, to form a cleft at the top of the heterodimer, in which is located the α subunit conserved sequence Asp-x-Asp-

x-Asp-Gly-x-x-Asp (Humphries *et al,* 1996). This sequence is thought to be involved in the divalent cation binding (Michisita *et al,* 1993; Mould *et al*, 1995) which is essential for integrin function, both in terms of affinity and specificity. Binding of an integrin to its substrate requires activation, and once activated and after association with the appropriate ligand, the integrins transmigrate along the plasma membrane giving rise to the association of a number of integrins and ultimately after cytoskeletal rearrangements, forming an organised complex termed the focal adhesion complex. Activation of the integrins by ligand binding is associated with a structural change in the cytoplasmic domain of both subunits (Lee Jie-O *et al,* 1995). This allows the recruitment of cytoskeletal and signalling molecules to the focal adhesion complexes.

Formation of focal adhesion complexes and the associated cytoskeletal organisation appears to be primarily controlled by signalling pathways in which the Rho family of small GTPases are essential (Miyamoto et al 1995; Hall 1998). This family of G-protein related molecules include Rho, Rac and cdc42. Rho was shown in response to extracellular ligands through a number of signalling events (Ridley et al, 1992), that activation of Rho leads to the assembly of actinomysin contractile filaments in 3T3 fibroblasts (Ridley et al 1992). This lead to the conclusion that Rho acted as a key participant in the signalling cascade which linked the membrane receptors to the cytoskeleton. Subsequent to this work two further small cytoskeletal GTPases were described, Rac which regulates the formation of lamellipodia and membrane ruffles, and cdc42 which when activated, causes actin-rich surface protrusions called filopodia (Allen et al, 1997; Hall 1998).

1.5.2 Integrin affinity

Integrins are regulated at both transcriptional and cell-surface expressional levels by a number of different factors. The specificity of different integrin heterodimers for different extracellular matrix components are well characterised - shown in Table 1.1 (Hynes, 1992). Some integrins are known to have their affinity for ligand modulated by intracellular events, which is known as "inside-out" signalling. Responses to the ECM mediated by integrins, so-called "outside-in" signalling, such as attachment dependent responses to growth factors or avoidance of apoptosis (Ruoslahti *et al,* 1994) have also been demonstrated in a variety of systems. However, the actual *in vivo* "outside in" signalling roles of each individual integrin heterodimer are less will delineated.

Gene targeting experiments of the individual subunits have revealed both severe and subtle phenotypes (Hynes, 1996). Embryonic lethal phenotypes have usually been associated with specific adhesive deficiencies, such as $\alpha5$ to fibronectin or $\alpha4$ to VCAM (Yang *et al,* 1995), both of which proved to be crucial in embryogenesis. The subtle phenotypes found in some other targeting experiments, such as $\alpha1$ (Gardner *et al,* 1996) and $\alpha7$ (Mayer *et al,* 1997) suggest that a group of integrins are concerned with sensing of the ECM without being absolutely required for structural integrity.

Table1.1: Integrin ligands and cellular expression

Integrin Dimer	***Ligand***	***Expression***
$\alpha1\beta1$	Collagen's I, III, IV, VI; laminin	FB, EC, SMC, T
$\alpha2\beta1$	Collagen's I, II, III, IV, VI; laminin	FB, EC, LK, Platelet
$\alpha3\beta1$	Collagen I, laminin, fibronectin, entactin	FB, EP, T
$\alpha4\beta1$	Fibronectin, VCAM-1; thrombospondin	LK
$\alpha5\beta1$	Fibronectin, thrombospondin	FB, EC, EP, P, T
$\alpha6\beta1$	Laminin	FB, EC, EP, P, T
$\alpha8\beta1$	?	EC, EP
$\alpha9\beta1$	?	EP
$\alpha10\beta1$	Collagen II	CH
$\alpha V\beta1$	Fibronectin	FB
$\alpha L\beta2$	ICAM-1, ICAM-2, ICAM-3	LK
$\alpha M\beta2$	ICAM-1, iC3b, fibrinogen, factor X	LK
$\alpha X\beta2$	Fibrinogen, iC3b	LK
$\alpha V\beta3$	Vitronectin, fibrinogen, fibronectin	FB, EC
$\alpha V\beta5$	Vitronectin	FB
$\alpha V\beta6$	Fibronectin	EP
$\alpha6\beta4$	Laminin	FB, EP
$\alpha4\beta7$	Fibronectin (CS-1), VCAM-1	T lymphocytes
$\alpha E\beta7$	?	IE lymphocyte
$\alpha IIb\beta3$	Fibrinogen, fibronectin, vitronectin	P
LRI	Fibrinogen, fibronectin, vitronectin, collagen (IV), von Willebrand factor	Neutrophil

IC3b=inactivated complement component; ICAM=intercellular adhesion molecule; VCAM=vascular cell adhesion molecule; FB=fibroblast; EC=endothelial cell; LK=leukocyte; EP=epithelial cell; SMC=smooth muscle cell; CH=chondrocytes; P=platelet. (Adapted from Mutsaers *et al*, 1997)

1.5.3 Collagen integrins

Four integrin heterodimers are known to be collagen receptors: $\alpha1\beta1$, $\alpha2\beta1$, $\alpha3\beta1$ and $\alpha10\beta1$ (Gullberg *et al*, 1992; Yamamoto *et al,* 1995; Camper *et al*, 1998). $\alpha2\beta1$ is predominantly epithelial in distribution, but also plays a role in signalling in fibroblasts (Wu and Santoro, 1994). $\alpha3\beta1$ is widely distributed and binds a broad range of ligands including fibronectin, laminin 5 (Carter *et al*, 1991) and collagen, along with cell surface protease systems (Xue *et al,* 1997). $\alpha10\beta1$, which was only recently characterised, is expressed on chondrocytes only (Camper *et al*, 1998). $\alpha1\beta1$, whilst having a dynamic pattern of expression in the embryo (Duband *et al,* 1992) is confined to mesenchymal and endodermal tissue in the adult, notably smooth muscle, fibroblasts, hepatocytes and the microvascular endothelium (Voight *et al*, 1995; Belkin *et al*, 1990). All receptors except $\alpha10\beta1$ are present on dermal fibroblasts, and all three contribute towards collagen binding (Gardner *et al*, 1996), although $\alpha3\beta1$ may serve only as an accessory role (DiPersio *et al*, 1996).

Targeted null mutations for $\alpha1$ and $\alpha3$ have been described; $\alpha1$ null mutant animals show no gross developmental deficits, but null fibroblasts show a specific deficiency in collagen dependent proliferation (Pozzi *et al*, 1998). $\alpha3$ animals are not viable beyond birth, and show deficits in epidermal basement membrane formation (DiPersio *et al*, 1997) and in branching morphogenesis in lung and kidney (Kreidberg *et al*, 1996). While fibroblasts from the $\alpha1$ null animal show striking absence of adhesion to collagen type IV, they show no appreciable deficit in adhesion to collagen I unless

they are functionally deficient in α2 or α3 (Gardner *et al*, 1996). The deficiency in collagen IV binding is unusual as type IV collagen is mainly located in and around the basement membrane, which is mainly populated with keratinocytes which are largely devoid of α1. The α1β1 null animal did not show any increase in expression of α2β1, which would suggest that there is no obvious compensation mechanism between these two receptors (Gardner *et al*, 1996 and 1999). However, it is possible that in these models, one arm of a finely tuned mechanism has been ablated, and henceforth the remaining α2β1, whilst expressed at normal levels, is somehow left uncontrolled, which may allow α2 signalling to play a role in the phenotype of these knockouts.

1.5.4 Integrin regulation by growth factors

Growth factors, as well as ECM proteins regulate the expression of integrins. The best characterised of these growth factors are TGFß and PDGF. TGFß1 upregulates ß1 and ß3 integrins as well as α1, α2 and α5 subunits (Enenstein *et al*, 1992; Heino *et al*, 1989) - elevating both protein and mRNA levels, assembly into corresponding αβ complexes and exposure on the cell surface of human fibroblasts. PDGF-BB also stimulates gene expression of integrin ß1 in swiss 3T3 cells (Bellas *et al*, 1991), α5 and ß3 in aortic smooth muscle cells (Janat *et al*, 1992) and α2 in human foreskin fibroblasts (Ahen and Kristofer, 1994). Furthermore, the effects of PDGF on integrin expression can also be altered by attachment to different matrices. Fibronectin-rich cultures and fibrin gels enhance PDGF-BB induction of α3 and α5 integrin subunit

mRNA. However, collagen gels attenuated these responses, whilst promoting maximal α2 mRNA expression (Xu and Clark, 1996).

The biosynthesis and assembly of αβ1 integrins is a multifactorial process in which distinct α subunits may have to compete for a limited supply of ß1 subunit, or vice versa, hence there are multiple ways of regulating these complexes in different cell types (Heino *et al,* 1989). These studies and others indicate that the ECM and growth factors are able to act in synergy in order to recruit the integrins required for the correct phenotype within a specific environment.

1.5.5 Integrins and mechanical load

A vital part of understanding the effects of mechanical load is determining the mechanism by which a mechanical signal can be transduced into a biochemical or cellular response. There is increasing information in the literature on the relationship between the ECM, integrins, signalling molecules and the cytoskeleton which would point to a role for integrins in the sense and transduction of a mechanical stimulus.

Regulation of integrin expression by load

There have been few studies to date assessing the effects of load directly on changes in integrin mRNA or protein expression. Studies on endothelial cells have demonstrated that 2-dimensional in vitro cyclical loading, whilst not affecting total

cell-surface expression of integrins, does alter the membrane clustering and organisation of subunits into focal adhesion complexes (Yano *et al*, 1997). Shear stress in vitro however, has been shown to induce increased α5β1 expression in endothelial cells, indicating that different directional forces can elicit specific effects at the cell surface (Girard *et al*, 1995). Many studies have highlighted changes in integrin expression within 3-dimensional *in vitro* systems, where cells are contained within a collagen gel. This system allows the analysis of cells in stress-free or contracting collagen gels, or attached, stressed matrices. Many studies have attributed the contraction of collagen gel by fibroblasts to the upregulation of α2β1, and subsequent decrease in levels of α1β1 (Klein *et al*, 1991; Riikonen *et al*, 1995). The effects of load on integrin lewvels are discussed in detail in Chapter 5.

1.5.6 Theoretical models of mechanotransduction

Despite the number of studies that have been conducted on the transduction of a mechanical response via integrins and the cytoskeleton, there is still little known about the direct mechanism of action, generating the final changes in cell phenotype. Many theories have been proposed by experts in the field based upon current understanding of cellular responses to stretch, and these models provide a summary of the current understanding of the involvement of integrins in a mechanical response.

One of the most developed theories on basic cellular mechanotransduction is that proposed by Donald Ingber. This hypothesis is known as Cellular Tensegrity, and

based on the tensional integrity of a cell, it describes the potential architectural basis of mechanotransduction (Ingber, 1993). Tensegrity is designed on the assumption that cells are physically structured to respond immediately to mechanical stress. Originally, it was assumed that membrane surface tension, osmotic force, cytoskeletal viscosity or molecular polymerisation controlled cell shape. Tensegrity, however, is a model based on the theory that cells stabilise by incorporating compression-resistant elements, either internal molecular struts or localised regions of extracellular matrix in order to resist the contractile forces of the cytoskeleton (Ingber *et al*, 1985). The elastic components of the cytoskeleton also assist in maintaining cellular structure and stability following exposure to stress.

This model can also provide a mechanical basis for the hierarchical structure of tissues, whereby cells are grouped in specific arrangements, and in turn contain smaller organelle in specific positions which exhibit their own mechanical stability (Ingber, 1997). This is demonstrated when a cell spreads on ECM on a culture dish, and the nucleus extends in parallel, polarising basally due to a stress concentration at the cell base (Ingber *et al*, 1987). This demonstrates that a local stress, which is applied to integrins may promote long-range structural rearrangements throughout the cell and nucleus. Similarly, mechanical stresses are not transmitted equally across the plasma membrane at points on the cell surface. Mechanical signals have been shown to converge on the integrin-containing focal adhesion complexes (FAC's) to activate a number of different signalling molecules (Ingber, 1991 and 1997). Recent reports indicate that cell structure, biochemistry and signal transduction can differ

dramatically depending on the level of mechanical forces across the complex (Chicurel *et al*, 1998; Maniotis *et al,* 1997; Choquet *et al*, 1997).

The majority of other proposed mechanotransduction models are formulated around a more conventional simple continuum hypothesis. These theories argue that the binding of integrins, as with any other classical receptor causes autophosphorylation of the transmembrane protein, or interaction with a cytoplasmic signalling molecule on binding of an external ligand. In these models however, the structure of the cell on binding is not addressed and indeed even a local stress induced by growth factors binding receptors can result in global structural rearrangements throughout the membrane protein. This may expose previously sequestered regions within the cytoplasmic tail, thereby changing binding affinity, and resulting in biochemical changes.

Other models suggest that the cytoskeleton responds to force and deformation in a highly localised manner, with little evidence for interconnections among cytoskeletal elements or differing cellular layers. A very recent study by Heidemann and colleagues (1999) supports this study and argues against the theory of the tensegrity structure. This model suggests that cells behave as a three-layer structure (Dong *et al,* 1991) with an elastic nucleus surrounded by viscoelastic cytoplasm all surrounded by an elastic actin shell with sustained tension. The latter allows the recruitment of actin to integrins upon application of mechanical load (Heidemann *et al,* 1999), which is widely postulated in many of the models of mechanical connectivity described.

However, the latter study, by using needle deformation of the membrane and/or cytoskeleton have shown only localised responses which is inconsistent with complementary force interactions between tensile actin and microtubules, previously predicted to promote widespread rearrangements (Heidemann and Buxbaum, 1998; Ingber *et al*, 1997).

These differing models of mechanotransduction and cellular responses to forces all highlight the diversity of effects stretch can exert upon a single cell. However, as described, all the models agree that mechanical stress is detected and transduced via cell surface receptors and that integrins are excellent candidates for this role. As the body of literature concerning the role of integrins in response to stretch expands, more information can be added to these models to further an understanding of biological mechanotransduction, and the consequences of it. This thesis aims to assess the effect of mechanical load on dermal fibroblast collagen deposition and organisation, and the role of integrins in these processes.

1.6 HYPOTHESIS OF STUDY

The hypothesis for this study is that mechanical load acting synergistically with growth factors associated with wound healing directly influence fibroblast function. The resultant effect is the enhanced synthesis, deposition and alignment of collagen. It is further hypothesised that mechanical stress activates specific integrins at the cell surface and is translated into a biochemical response via these receptors.

1.7 AIMS OF THIS THESIS

The focus of the project is the determination of the effect of mechanical load on dermal fibroblast procollagen deposition using two *in vitro* systems of exerting an external mechanical force. A 2D loading system will be used to examine the effects of load and growth factors on procollagen synthesis and processing. A 3D loading system will be used to assess the effect of matrix and load on collagen deposition, applied in a more physiological setting. The study will then begin to assess some of the fundamental mechanisms involved in these responses.

The principal aims of this thesis are:

- To explant, culture and characterise human dermal fibroblasts (HDF)
- To assess the responses of HDF procollagen synthesis to mechanical loading in the 2D system and the effects of serum, TGFß and thrombin on this response.

- To investigate the effect of mechanical loading on the processing of procollagen into insoluble collagen and the role of procollagen c-proteinase (PCP) in this response.
- To assess by SEM the role of dermal fibroblasts and mechanical load on collagen fibril alignment within 3D collagen gels.
- To develop a radioactive method to detect and quantitate collagen synthesis and deposition in a 3D collagen gel in order to detect any changes in synthesis and deposition in response to load.
- To investigate the consequences of blocking collagen synthesis and degradation on collagen gel alignment in response to load.
- To determine the effects of 2D load on integrin expression, regulation and the role of specific integrins in mechanotransduction.

CHAPTER TWO

Materials and Methods

2.1 MATERIALS

All reagents were obtained from BDH/Merck (Lutterworth, UK) unless otherwise indicated. All water used for the preparation of buffers, solvents used for the preparation of HPLC buffers and solutions and HPLC columns and pre-columns was MilliQ filtered. Sterile tissue culture plasticware was obtained from Falcon and Bibby-Sterilin, Marathon Laboratory Supplies (London, UK). All 2-dimensional loading equipemtn (Flexcell, Flex I plates) was obtained from Flexercell International Co. (NC., USA). Sterile cryotubes, 8-well chamber slides (Nunc), Dulbecco's Modification of Eagles Medium (DMEM), Dulbecco's Modification of Hams F12 medium (DME Hams F12 medium), trypsin, antibiotics, L-glutamine and fetal calf serum (FCS) were all purchased from GIBCO-BRL (Paisley, Scotland, UK). The same batch of FCS was used for all the experiments described. BSA, holo transferrin, DMSO, L-ascorbic acid, proline, hydrogen peroxide (H_2O_2), NBDCl, monoclonal mouse anti-myosin (smooth), anti-α smooth muscle actin (α-SMA) and anti-vimentin antibodies were all purchased from Sigma Chemical Company (Poole, Dorset, UK). Monoclonal mouse anti-desmin and rabbit anti-human von Willebrand Factor antibodies were purchased from Dako Ltd (High Wycombe, Bucks., UK). Recombinant human TGF-β1 was obtained from R&D Systems Ltd (UK). Type II collagenase was obtained from Lorne Laboratories (Twyford, Reading, Berkshire, UK) and Type I collagenase from Sigma Chemicals Co. (Dorset, UK). Carbon dioxide (CO_2) and helium gases were purchased from BOC (London, UK). Mycoplasma detection kit was purchased from Gen-probe (San Diego, USA). Human umbilical vein endothelial cells (HUVEC), endothelial cell basic medium (EBM) and

supplements, human recombinant epidermal growth factor (hEGF), hydrocortisone, gentamycin/amphotericin B, bovine brain extract (BBE) and FCS were all obtained from Tissue Culture Services (Botolph Claydon, Buckinghamshire, UK). Rat kidney fibroblasts (NRK-49F) were obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, UK) and rat thoracic aorta smooth muscle cells (A10) from the American Type Culture Collection (Rockville, Maryland, USA). Normal serum (horse and goat), biotinylated anti-rabbit immunoglobulin G (IgG) and biotinylated anti-mouse IgG, Vectastain Elite ABC Reagent and 3,3'diaminobenzidene (DAB) were all purchased from Vector Laboratories, (Peterborough, UK). Aquapolymount was purchased from Park Sciences (Moulton Park, N.H., USA). Activated charcoal was purchased from Hopkins and Williams (Essex, UK). Vacuum filtration unit and all filters were purchased from Millipore (Watford, Hertfordshire, UK). Titertek multiscanner (microtitre plate reader) was purchased from ICN Flow (High Wycombe, Bucks, UK). Dry-block sample concentrator (Techne DB-3 SC-3) and sample concentrator (Savant Speedvac Plus SC110 AR) were obtained from Life Science International (Basingstoke, Hampshire,UK).

2.2 METHODS

2.2.1 Isolation, and culture of human dermal fibroblasts.

Human dermal fibroblasts were routinely cultured following explant isolation of tissue taken from breast reduction operations. Under sterile conditions, the tissue was chopped finely and rinsed several times in the collection media to remove traces of blood. The tissue was then placed into 10cm petri dishes, previously scored to generate grooves for gripping, and covered with 2mls of DMEM supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml), L-glutamine (4 mM) and FCS (20%). Explants were incubated in a humidified atmosphere of 10 % CO_2 in air at 37 °C for 16 hours. After this time, 10mls more media was added onto the explants, and left for 4-5 days. The media was routinely changed every 5 days for up to 6 weeks. After this a time, pieces of remaining tissue were removed from the dish and the cells allowed to grown until confluence. At confluence, cells were trypsinised from the dish using trypsin/EDTA. Cells were then plated into a 75 cm^2 culture flask and incubated in a humidified atmosphere of 10 % CO_2 in air at 37 °C. The cells were maintained under standard tissue culture conditions, described below. Upon reaching visual confluence the cells were designated to be passage 1.

The fibroblasts were used between passages 6 and 12. Cells were maintained in 10% fetal calf serum (FCS) and routinely passaged upon reaching confluence. All fibroblast cell lines were subjected to characterisation by immunohistochemistry in order to screen for possible contamination from smooth muscle cells and endothelial

cells, determined by immunohistochemistry using the Vectastain ABC kits as per manufacturers instructions. Cells were characterised by the detection of desmin, vimentin and Von-willebrands Factor antigens compared to control cultures of endothelial cells and smooth muscle cells from cell lines (ATCC). Cells were also routinely tested for mycoplasma contamination using a probe for mycoplasma ribosomal RNA (Gen-Probe Mycoplasma T.C.II, Lab Impex, Teddington, UK). Tests were always negative in every cell line tested.

Routine cell culture

Cells were routinely grown in 75 cm^2 triangular flasks in DMEM/ 10% FCS. The cultures were incubated in a humidified atmosphere of 10 % CO_2 in air at 37 °C. Each cell line was routinely checked for mycoplasma contamination at monthly intervals using a commercially available mycoplasma detection kit. Culture medium was changed every four days and cells were passaged 1:2 or 1:4 upon reaching visual confluence.

Cell passage was performed by removing the culture medium, washing once with 10 ml PBS, and adding 2 ml of trypsin/EDTA (trypsin 0.05 % w/v, EDTA 0.02 % w/v) to the cell layer. The cells were then incubated at 37 °C until detachment from the tissue culture plasticware occurred (approximately 3 min). Detachment from the plasticware was observed under an inverted light microscope (Axioscop 20, Carl Zeiss, Germany). 8 ml DMEM/10 % FCS was then added to the flask to inactivate the trypsin and 5 ml (1:2 passage) or 2.5 ml (1:4 passage) of this suspension was added to fresh culture

flasks. The volume in each flask was made up to 10 ml by further addition of DMEM/10 % FCS.

Storage of primary cell cultures

Freezing of cells

Primary cultures were frozen for storage and thawed as needed. All fibroblasts for storage were pooled in DMEM/20 % FCS at a ratio of 1.5 ml media/confluent 75 cm^2 flask. An equal volume of DMEM/20 % FCS containing 20 % DMSO was added dropwise to the cells. 1.5 ml aliquots of this mixture, representing ½ of a confluent flask, was added to sterile cryotubes and frozen overnight at -70 °C in a polystyrene container packed with paper towels to permit slow freezing. Tubes were then removed and stored in liquid nitrogen.

Thawing of cells

A single tube of cells was removed and thawed at 37 °C in a water bath. The contents were then removed and transferred to a sterile 50 ml centrifuge tube. 8 ml DMEM/20 % FCS was added dropwise to the cell suspension with continual agitation. The cells were then spun down at 3000rpm for 5 min, the supernatant discarded, the cells re-suspended in 10 ml DMEM/10 % FCS and plated into a 75 cm^2 culture flask. Routine cell culture was then carried out as described above. Passage and population doubling (PD) numbers were increased as appropriate to the thawed cells.

Immunohistochemical characterization of primary fibroblast cultures

The fibroblastic nature of primary cells was determined by immunofluorescence staining of cell layers with various antibodies to cytoskeletal and surface proteins. The staining pattern exhibited by the primary cells was compared with that obtained in various cell lines employed as controls. Every primary cell isolation prepared and used in the experiments described below was characterized immunohistochemically. Primary cell cultures and a rat thoracic aorta smooth muscle cell line (A10s) were plated in DMEM/ 10 % FCS and incubated in 10 % CO_2 as described above. A rat kidney fibroblast cell line (NRK-49F) was plated in DME Hams F12 medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), amphotericin B (0.25 μg/ml) and 10 % FCS and incubated in 5 % CO_2. A human umbilical vein endothelial cell line (HUVEC) was plated in EBM supplemented with hEGF (0.01 ng/ml), hydrocortisone (0.001 mg/ml), gentamycin (0.05 mg/ml), amphotericin B (0.05 μg/ml), BBE (0.012 mg/ml) and FCS (2 %).

At confluence, cells were trypsinized as described above and plated in their respective media into 8-well chamber slides and incubated until sub-confluent. Cultures were washed twice with PBS, fixed in cold (-20 °C) methanol at -20 °C (cytoskeletal proteins) or paraformaldehyde (4 % w/v) (surface proteins) at room temperature for 3 min. Cells were then washed three times with PBS and stored at 4 °C in PBS prior to staining. Slides were incubated with 3 % H_2O_2 in PBS (v/v) to block endogenous peroxidase activity, rinsed three times in PBS and non-specific binding was blocked with non-antigenic serum (which was prepared from the species in which the

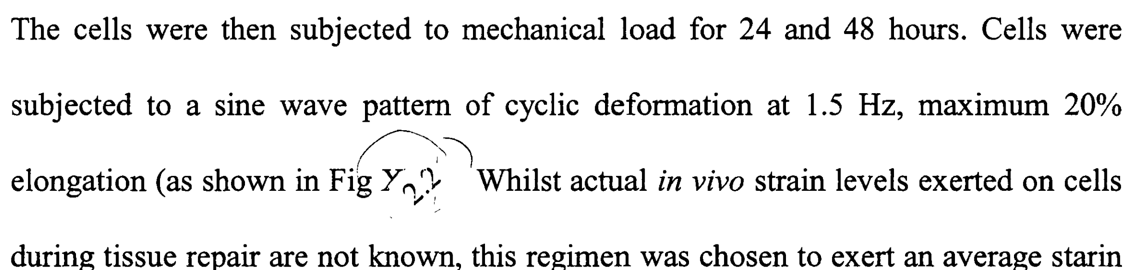
secondary antibody is made). The cells were then incubated with individual primary antibodies diluted in PBS for 45 min at ambient temperature: anti-vimentin (1:40, clone V9), anti-α-SMA (1:1000, clone 1A4), anti-desmin (1:100, clone DE-R-11), anti-myosin (1:1000, clone hSM-V) and anti-von Willebrand Factor (1:1000). Cells were subsequently incubated with biotinylated secondary antibody (against the animal that the primary antibody was raised in: anti-rabbit IgG for anti-von Willebrand factor and anti-mouse IgG for all other antibodies) for 60 min at room temperature. The avidin-biotin complex was visualized using DAB as a chromogen. Cells were washed twice in water, counterstained with haematoxylin and mounted with Aquapolymount.

2.2.2 TWO-DIMENSIONAL MECHANICAL LOADING

2-Dimensional Mechanical Loading

Cells were 2D mechanically loaded using the FX3000 Flexercell Strain Unit (Flexcell Int. Corp., McKeesport, PA) Fibroblasts were plated on 6-well flexible bottomed type I collagen-coated Flexplates® in 10% FCS and grown to confluence (approx. 3 x 10^5 cells/well) (well size of $4.6cm^2$). The media was then changed to 0% FCS pre-incubation media containing BSA (1mg/ml) and transferrin (1mg/ml), which was in turn replaced by incubation media (0% or 10% FCS or growth factor) after 24 hours. Plates were then placed into a rubber manifold and a CO_2 incubator. Negative air pressure was applied beneath the plates by a pump, and controlled with respect to strain and frequency by a computer and as series of valves connected to the hard drive. The negative air pressure causes the membranes to dome downwards, which increases the surface area of the membrane and therefore applies mechanical load to the attached cells. A gradient of load exists across the membranes, with maximal load 3mm from the edge of the plate, and minimal load in the centre. However, an average is taken across the entire membrane for cell activity in response to load. These differences are taken into account only in immunohistochemical localisation or visual staining on the membrane.

The cells were then subjected to mechanical load for 24 and 48 hours. Cells were subjected to a sine wave pattern of cyclic deformation at 1.5 Hz, maximum 20% elongation (as shown in Fig Y2.2) Whilst actual *in vivo* strain levels exerted on cells during tissue repair are not known, this regimen was chosen to exert an average starin



level on the dermal fibroblasts. As movement is always occurring during wound healing, a cyclical strain was applied as opposed to a static one to exert differential loading on the cells throughout the course of any experiment. The flexible-bottomed wells in this system are also subjected to differential strain across the well on application of emchancial loading. The empirical measurement of surface deformation is shown in Fig 2.1 Unloaded wells were set up in an identical fashion to provide a control for comparison. A diagrammatic representation of this system can be found in Fig. 2.3

Fig. 2.1: Surface deformation of Type I Flexplates

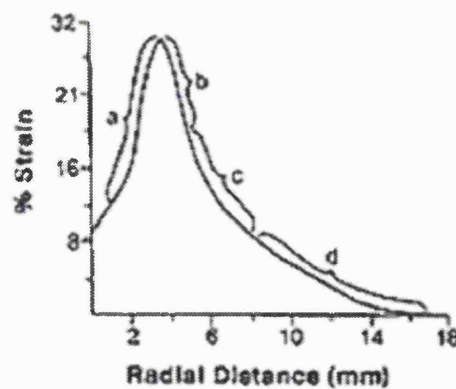
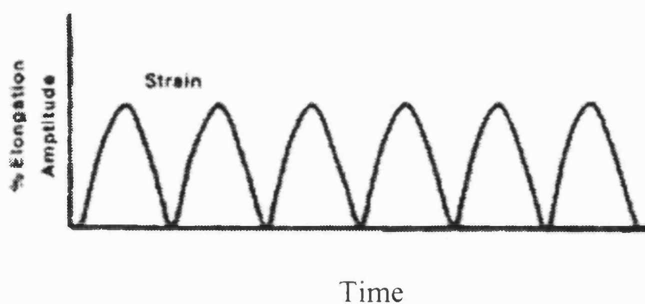
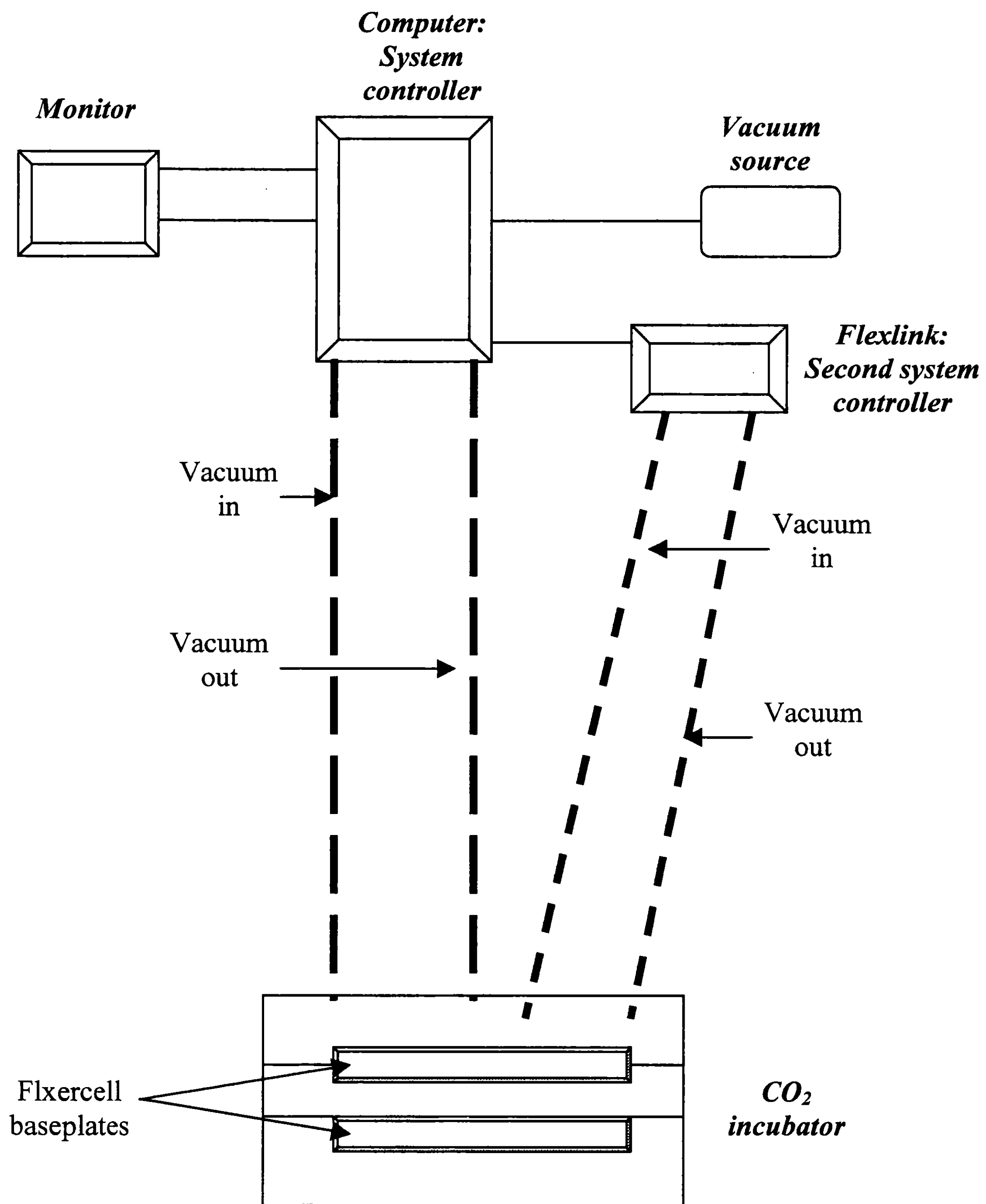


Fig 2.2: Typical loading regimen used in 2-dimensional system.



Time

Fig. 2.3: Diagrammatic representation of the Flexercell Strain Unit.



2.2.3 Measurement of Fibroblast Procollagen Synthesis

Principle of the assay

Procollagen metabolism by cultured fibroblasts was assessed by measuring hydroxyproline (OHpro) in both the media and cell layer separately. Intact proteins (ethanol insoluble fraction), as well as in low molecular weight degradation products, derived from procollagen synthesized and degraded during the culture period (ethanol soluble fraction) were analysed using a HPLC method previously developed in this laboratory (Campa *et al*, 1990, McAnulty *et al*, 1991). The principle steps involved in measuring procollagen metabolism are shown in Figure 2.4 and given in more detail below.

Hydorxyproline represents approximately 12 % of the primary sequence of procollagen (Laurent *et al*, 1981) and is essential for the formation of the collagen triple helix. This imino acid, however, is not present to a significant level in most other proteins. Other proteins containing OHpro are elastin, C1q component of complement, acetylcholinesterase, surfactant apolipoproteins A and D and ficollin α and β. These proteins have not been demonstrated to be produced by isolated fibroblasts in culture and therefore OHpro concentration from fibroblast cultures *in vitro* is an excellent determinant of procollagen metabolism.

Assessment of procollagen in the 2D Culture System

Procollagen synthesis was estimated in confluent cultures (grown in 10% FCS) of fibroblasts grown in Flexplates®, using methods developed by this lab (Campa *et al*, 1990). Cells were incubated for 24 hours in serum-free media supplemented with proline (0.2mM), ascorbic acid (50mg/ml), BSA (1mg/ml) and transferrin (1mg/ml). The media was then changed to an identical one containing growth factors or serum. Cells were incubated for 24-48 hours with or without mechanical load. For incubation times over 24 hours, the media is supplemented every 24 hours with ascorbic acid in order to ensure efficient hydroxylation of proline and lysine and thus maintain linear kinetics for procollagen synthesis. Ascorbate can directly stimulate procollagen synthesis, both at transcriptional and post-translational levels (Geesin et al, 1988; Kirata et al, 1991). Ascorbate has been shown to stimulate collagen synthesis in dermal fibroblasts without stimulating changes in other non-collagenous protein production (Murad et al, 1983). It also acts as a co-factor for prolyl hydroxylase, which acts to hydroxylate proline residues within the new procollagen molecule. This step is essential in part for collagen triple helix formation to occur (Steinmann et al, 1981).

Sample processing

The media was then removed and the cell layer washed with 1ml of PBS, which was added to the original medium sample. Ethanol was added to a final concentration of 67% and left at 4^0C overnight to precipitate intact proteins. The cell layer was then scraped into 1ml of PBS and the plate washed with 1 ml of PBS. This was again

precipitated overnight with 4mls 100% ethanol. Both cell layer and media samples were then treated identically: The precipitated solution was filtered on a Millipore Filter Unit through 0.45mm pore-sized filters. The resultant filtrate containing the free OHpro (representing procollagen degradation) was collected and dried on a hot-block. Filters containing the proteins were transferred to hydrolysis tubes, and both filters and dried supernatant were suspended in 2ml 6M HCl for hydrolysis at 110^0C for 16 hours. Hydrolysates were decolourised with activated charcoal and centrifuged at 2500rpm for 15 minutes. Clear supernatant was then aspirated prior to chromatography. 100ml of sample hydrolysate was dried and derivatised using 7-chloro-4nitrobenzo-2-oxa-1,3-diazole (NBDCl). This was then measured using reverse-phase HPLC (Beckman System Gold, Beckmann, High Wycombe, Bucks, UK).

Quantitation of OHpro content in each 100ml sample injected into the column was determined by comparing peak areas of chromatograms obtained from each sample to those generated from the two standard solutions derivatized from two OHpro standards. All values obtained for the ethanol-insoluble fractions were corrected for the amount of OH pro in the media and cell layer separately at time 0. These values represent the procollagen protein production (procollagen synthesised and present as intact procollagen molecules in both the media and cell layer) and procollagen degradation (procollagen synthesised, subsequently degraded and present as OHpro or small peptides in the supernatant).

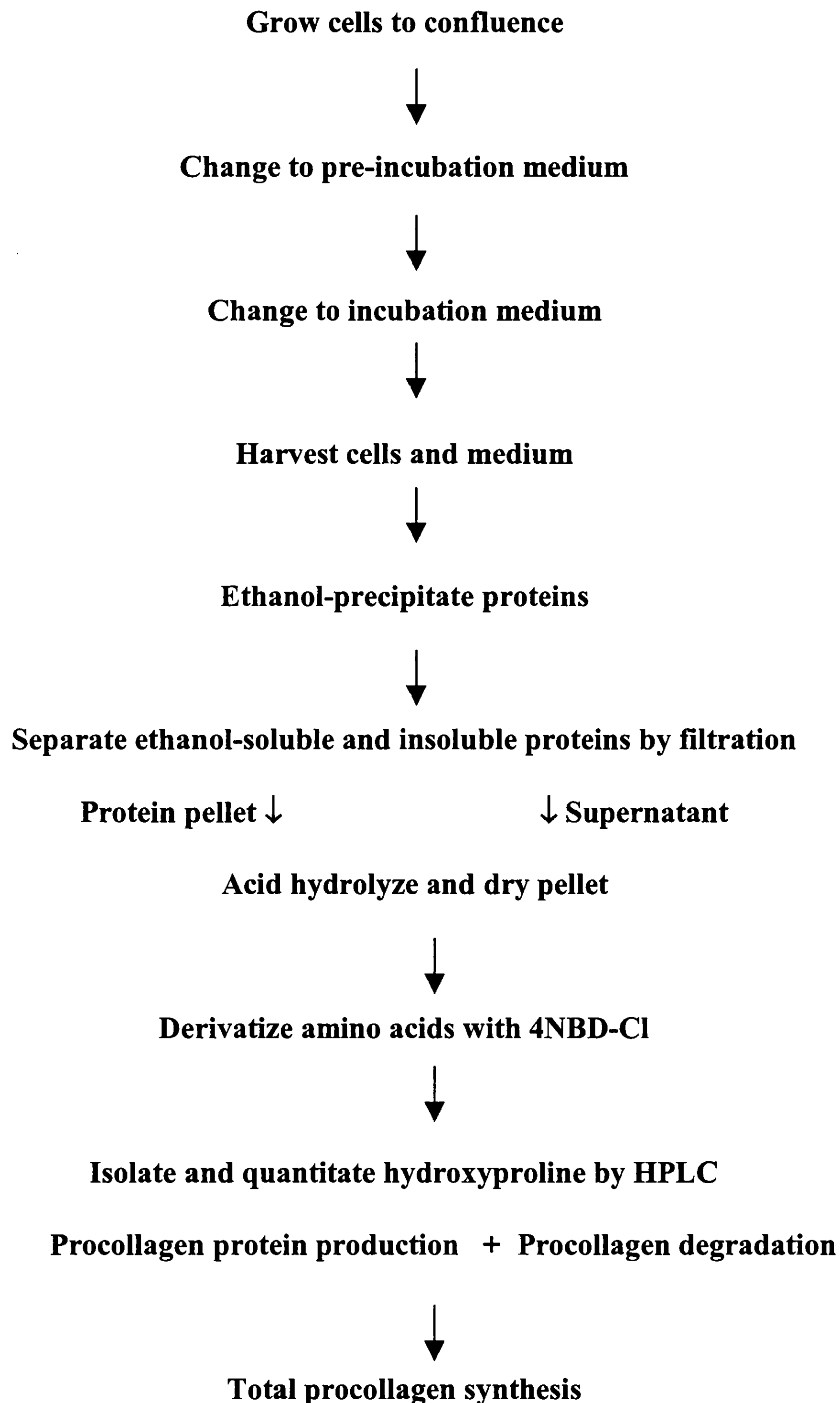
For each procollagen metabolism experiment performed, parallel cultures (n=3) were included for estimation of cell number by direct cell counting. After the incubation period, medium was removed, the cells washed with 1ml PBS and trypsinized from each well by addition of 100µl trypsin for 3min. Where a single-cell suspension was

difficult to obtain, 100µl of type II collagenase (3000 units/ml) was also added to dissociate the cells. A 10µl aliquot of the resulting suspension was then counted, using a haemocytometer, as before. This allowed estimation of cell replication and correction of the procollagen synthesis values for final cell numbers in the cultures.

The value obtained represent the procollagen production (procollagen synthesised and present as intact procollagen molecules in both the media and cell layer separately) and procollagen degradation (procollagen synthesised, subsequently degraded and present as OHpro or small peptides in the media). The sum of these two values represents the total procollagen synthesis over the incubation period by the fibroblasts. Parallel cultures included for cell counts enabled calculation of the procollagen metabolism per cell. Data is expressed as total procollagen synthesis, procollagen production, and procollagen degradation with units of nmoles OHpro. Data for procollagen degradation is also expressed as the proportion (or percentage) of total synthesis by the calculation:

$$\frac{\text{EtOH-soluble OHpro}}{(\text{EtOH-soluble} + \text{EtOH-insoluble})} \times 100 = \%\ \text{procollagen degradation}$$

Fig. 2.4: Flow diagram showing principal steps in assessing procollagen production by cells in monolayer culture



2.2.4 Measurement of total non-collagenous protein synthesis

Cultures were set up as described above for HPLC procollagen determination assay in the 2D loading system. Phenylalanine is used in the determination of non-collagenous protein as this amino acid is not present in collagen protein, but is found in abundance in the large majority of other cellular proteins in eukaryotes. Therefore, it provides a reliable marker of total cellular protein synthesis, without including that fraction of synthesis that is taken by procollagen, thus allowing the separation of collagenous and non-collagenous protein synthesis.

Total protein production was determined by adding 5μCi/well ^{3}H-phenylalanine at the beginning of the experimental incubation period. Total non-collagenous protein synthesis was assessed in control and loaded cultures stimulated using 10% serum, TGF-ß and thrombin at 24 and 48 hours. Samples were then treated and hydrolysed as described for HPLC methodology as before. 20μl of the hydrolysate was placed in a scintillation vial with 3mls of liquid scintillant. The levels of radioactive incorporation into the protein were then measured on a ß-scintillation counter.

2.2.5 Northern analysis of mRNA

Procollagen types I and III mRNA levels were be measured in the 2D loaded and control cells under each of the test conditions following isolation of RNA by the TRIzol method (Gibco, Paisley, UK) according to the manufacturers directions. Six wells were used for each determination. RNA was isolated from total cell lysate in 3 separate stages. Samples were shaken with chloroform, centrifuged for 15mins, and the supernatant transferred to a new eppendorf. This fraction was then shaken with isopropyl alcohol and centrifuged for 10mins. The RNA pellet was washed with 75% ethanol, re-centrifuged for 5 minutes and left to airdry. When the pellet was dry, the RNA was resuspended in 0.5% SDS and quantitated using a spectrophotometer. Northern analysis is then performed on the samples using the method previously described by this lab (Campa, Bishop and Laurent, 1991).

A 2µl aliquot of RNA was quantitated in 1ml of DEPC water at 260nm and the volume of sample required for loading of 5µg total RNA was calculated. This volume of RNA was mixed with double the volume of RNA loading buffer (Sigma, UK) containing Ethidium Bromide (EtBr) and heated to 65^0C for 10 minutes prior to loading onto the gel.

Samples were loaded onto a 1% agarose gel and run at a constant voltage of 80V in 1X MOPS running buffer for approximately 3 hours. The integrity of the RNA, and uniformity of loading was confirmed by the presence of the 18S and 28S ribosomal RNA bands identified by EtBr staining. RNA was transferred to a nylon membrane by Northern blot in 20X sodium chloride/sodium citrate solution (20X SSC) overnight.

The transferred RNA was then cross-linked to the nylon membrane using a UV crosslinker.

Membranes were pre-hybridised in a solution of 5X Denhardts solution, 5X SSC, 0.1% SDS containing 100ml/ml salmon sperm DNA (Gibco BRL) for 45 mins at 65^0C. Probes for procollagen a_1(I) (ATCC), PCP and PCPE (kindly donated by Prof E. Kessler of University of Tel Aviv) were radioactively labelled using ^{32}P d-CTP (Amersham). The labeled probe was added to the hybridisation solution and the filter incubated at 65^0C overnight. After this time the filter was removed and washed in 2X SSC, 0.1% SDS for 10 minutes at room temperature, followed by a high stringency wash in 0.2% SSC, 0.1% SDS for 1 hour at 65^0C. The membrane was wrapped in Saran Wrap and exposed to film until a suitable signal was visible. The generated signal was then quantitated using a laser scanner (Sharp JX 330) linked to Imagemaster ID software (Pharmacia). Absorbance values of the signals representing the specific probe size were normalised relative to the loading of total RNA in the same sample.

2.2.6 Western blotting analysis of protein

Antibodies

Antibodies for western analysis

Primary antibodies for western blotting of PCP and PCPE were a kind gift from Professor Efrat Kessler (University of Tel Aviv). Polyclonal antibodies to the 36 kDa form of mouse PCPE (Kessler and Adar, 1989) were raised in rabbits and immunopurified. Rabbit antibodies to PCP (BMP-1) were directed to a synthetic peptide, AYDYLEVRDGHSESSTLIGRYC, corresponding to positions 489-510 in human pre-pro BMP-1/mTld linked to keyhole limpet hemocyanin. These antibodies were immunoaffinity purified on a peptide coupled to a sulfo-linked resin via the cysteine residue. Both antibodies were used at a concentration of 1:1000 for detection of proteins on nitrocellulose membranes.

Antibodies for immunoprecipitation

Specific antibodies were used for integrin subunit immunoprecipitation. Antibodies to α2 (P1E6), α5 (P1D6) and ß1 (P4C10) were all obtained from Gibco Life Sciences Ltd (Paisly, UK). Antibody to α1 (05-246) was obtained from Upstate Biotechnology Ltd (TCS, UK). All antibodies were monoclonal anti-human, raised in nude mice. Antibodies were individually optimised for immnuoprecipitation, and were used between 1:80 and 1:100 for precipitation of proteins from lystaes.

Antibodies for functional blocking

A number of antibodies were used for function blocking of specifci integrins on dermal fibroblasts. All antibodies were obtained from Chemicon International Inc. (CA., USA). $\alpha1\beta1$ (MAB1973), $\alpha2\beta1$ (MAB1998) and $\alpha5\beta1$ (MAB1969) were all used at concentrations of 1:100 for immunoprecipitation. All antibodies were monoclonal mouse anti-human, and were purified immunoglobulin from protein A chromotagraphy.

Protein assay

Protein levels in cell lysates and media samples were quantitated previous to western blotting and immunoprecipitation to control for total protein loading.

5μl of each lysate/media sample was placed into individual wells of a 96-well plate, and 25μl of reaction mixture was added to each well. 8 BSA protein standards of concentrations ranging from 2000μg/ml to 50μg/ml were also present on each plate. Samples were incubated with the reaction mixture for 30 minutes at 37^0C, and the plates were then read on a spectrophotometric plate reader (ICN Flow, Bucks, UK) at a wavelength of 580nm to quantitate colour changes of the reaction mixture between samples. The reaction mixture used was BCA (Bicinchromicic acid) which is a detection reagent for Cu ions (BCA protein assay kit, Pierce, Rockford, US). Binding of BCA to 2 Cu ions forms a complex which is purple in colour and has a strong absorbance at around 562nm. Total protein content in each sample was then calculated by comparing numerical readouts with the BSA standard curve of protein concentration generated within the same 96-well plate.

Sample preparation and electrophoresis

Media samples were removed from monolayer cultures and frozen at -20°C until required. Cell layer samples were lysed into RIPA buffer containing protease inhibitor cocktails (XXXXX). 20µl of media was added to 40µl of 2xSDS sample buffer (Novex, UK) and 2µl of ß-mercaptoethanol, and heated to 90^{0}C for 5 minutes prior to loading.. Samples of both cell layer and media were subjected to SDS PAGE as previously described (Laemmli, 1970) and transferred to nitrocellulose membranes.

SDS PAGE and blotting

Polyacrylamide gels were set up containing 8% acrylamide in the resolving gels and 4% in the stacking gels. 25µg of total protein was loaded per well for standard protein analysis. A broad range molecular weight marker was also run alongside all samples in every gel (Biorad, UK)> gels were run at 125V constant for 90 minutes. Gels were then removed from the cassettes and transferred onto a similar sized piece of Whatmann 3M blotting paper. A sheet of nitrocellulose (Hybond, Amersham Pharmacia, UK) was placed on the opposite side so as to be in contact with the gel, and 2 further pieces of blotting paper were placed on top of this to form a "sandwich" effect. This was then placed in between 2 sponges soaked in transfer buffer (20% methanol, Tris base, glycine). The sponges and gel were placed into the Novex Blot Module (Novex, UK) and transferred in 500ml of buffer at 25V constant for 90 minutes. After thistime, all protein had migrated from the gel onto the nitrocellulose membrane, and remained bound there.

Membrane treatments

After transfer, the membranes were washed with 10ml tri-buffered saline (TBS) for 5 mins at room temperature. The membrane was incubated with 10ml of blocking buffer (typically 3% skimmed milk in TBST) for 1 hour at room temperature. Membrane and primary antibodies to specified protein of interest were then incubated together in 10ml primary antibody dilution buffer and left overnight at 4^0C, shaking gently. The membranes were then washed 3 times for 5 mins each in TBS-tween (TBST), and incubated with HRP-conjugated secondary 10ml of blocking buffer with gentle agitation for one hour at room temperature. Membranes were washed in TBST as before. Proteins were detected by incubation of membrane with 1ml of ECL reaction mixture and gentle agitation for 1 min at room temp. Membranes were then wrapped in Saran Wrap and exposed to X-ray film for 10-60 seconds.

Immunoprecipitation and quantitation of biotinylated cell surface proteins

Biotinylation of cell-surface proteins:

Cells were mechanically loaded as described, in the presence or absence of serum/TGFß1 for up to 48 hours. At the end of the incubation period, cultures were trypsinised using a solution of trypsin/EDTA for 2 minutes at 37^0C. Trypisinisation was neutralised using 2 mls of DMEM, and the suspension of cells from 3 wells were pooled and centrifuged at 1500rpm for 5 minutes. The cell pellet was resuspended twice in 2mls of cold PBS and re-centrifuged. Once washed, the pellet was suspended in 2mls of a solution of 1% Biotin (Pierce, UK) in PBS and left for 1 hour at 4^0C with occasional agitation. Following biotinylation, the solution was centrifuged and washed in PBS as before. Cells were then suspended in 0.5ml of RIPA lysis buffer containing 1% SDS/1% NP-40 and a cocktail of protease inhibitors. Samples were left to lyse for 5 minutes at room temperature. Lysed samples were centrifuged at 2500 rpm for 5 minutes to pellet any insoluble protein material. Total protein in the resulting solutions were then quantitated using the Biorad protein assay.

Immunoprecipitation:

Following total protein quantitation, 300mg of total protein from each sample was aliquoted into an eppendorf. Antibodies to specific integrin subunits (see Antibody section for details) were then added to the lysates at a final concentration of 1:100. Samples were then placed into an eppendorf rotator, and left in a cold room at 4^0C for 3 hours to allow antibody binding. Following this, 10µl per sample of Protein G-

Agarose conjugated beads (Affinity Chromatography, San Diego, USA) were added to each eppendorf and once again left rotating at 4^0C for 3 hours to allow antibody-Protein G binding. Immunoprecipitation was terminated following this by centrifuging samples at 2500 rpm for 5 minutes at 4^0C. This centrifugation step precipitated the protein G-antibody complexes. Supernatants were aspirated and discarded, and the pellet washed in cold RIPA lysis buffer (as before) and re-centrifuged twice. The final pellet was then resuspended in 40µl of Tris-Glycine SDS-PAGE sample buffer (Novex, UK).

SDS-PAGE

SDS-PAGE gels were prepared as described in the previous section (see Western Blotting). A 10% resolving and 4% stacking gel were used for separation of immnuprecipitated samples. Gels were placed into the tank with 500mls of Tris-Glycine running buffer. Protein samples were heated at 90^0C for 3 minutes prior to loading onto the gel. Samples were centrifuged briefly to facilitate protein-bead separation and only the supernatant was loaded onto the gel. 10µl of molecular weight protein standard (Novex, UK) were also loaded onto each gel as a comparative marker. Gels were run at a constant 120V for 2 hours. Following running, gels were removed from the casing and blotted onto nitrocellulose as described in the previous section (see Western Blotting). Following blotting, membranes were blocked in 3% skimmed milk (Merck BDH Ltd, UK) in TBST at room temperature for one hour. The blocking solution was then replaced with an identical solution of 3% milk in TBST, but also containing a streptavidin-HRP conjugated secondary antibody at a final dilution of 1:1000 (Dako, UK). The secondary antibody was left to incubate with the

membrane for 1 hour at room temperature. This enabled the detection of only the cell-surface biotinylated integrin subunit expression in each sample. Membranes were then washed three times in TBST for 5 minute each. Blots were then incubated with ECL reaction reagents (Amersham, High Wycombe, UK) for one minute and excess fluid was drained from the blot. Membranes were wrapped in saran Wrap and exposed to ECL hypersensitive film (Amersham, UK) for up to 5 minutes.

Membranes were then stained using 10mls of undiluted Ponceau Red solution (Sigma, UK) in order to stain up for total immunoprecipitated integrin subunit in the sample. Both cell-surface and total protein bands were scanned in and quantitated using densitometric quantitation (Sharp laser scanner, Munich, Germany).

2.2.7 Confocal microscopy of fluorescence-labelled proteins

Following treatment, media was removed from each well of the Flexplates and the cell layer was washed using 1ml/well of cold PBS. Cells were then fixed and permeabilised within the wells by incubating in 500μl/well of 4% paraformaldehyde for 30 minutes at 4^0C. Wells were then washed twice in cold PBS and the wells were blocked using 0.5 ml of 1% BSA in TBS for 1 hour at room temperature. Blocking solution was removed and primary antibodies directed against specific integrins were added to the well in 1% BSA and left to bind for 2 hours at room temperature. Negative controls using PBS only in place of primary antibody were also set up to control for any non-specific antibody binding. This solution was then removed and the cells washed three times with TBST for 5 minutes each. All cultures were then incubated with a FITC-conjugated secondary antibody at a final dilution of 1:1000 in 1% BSA for 1 hour at room temperature. Cells were then re-washed three times for 5 minutes in TBST to remove excess secondary antibody. 19mm diameter coverslips (BDH, UK) were then placed onto each well over a drop of immunomount containing antifade solution and allowed to dry at 4^0C overnight.

Cells were then visualised using confocal microscopy (COMPANY?) at 630x magnification using a water immersion lens.

2.2.8 Antisense oligonucleotide treatment:

Principles of antisense technology:

Antisense oligonucleotides (OGN) are a relatively new method of specifically inhibiting new synthesis of individual proteins. By definition, the mRNA is the "sense" strand and any complementary sequence is 2antisense" to it. Since the antisense orientation is a pre-requisite for sequence-specific inhibition of translation, this term is attributed to the technique as a whole. This technique is based on naturally occurring gene regulation in bacteria, whereby new translation is inhibited by binding to the complementary sequence in the promoter region of a bacterial gene. Generally, any sequence may be targeted by antisense oligonucleotides (oligos). Even highly homologous members of a gene family may be inhibited selectively.

At present, 4 different mechanisms of antisense inhibition of gene expression are understood.

1. Oligos directed against the coding region of the mRNA arrest protein elongation by a steric block of the ribosome. Furthermore, oligos directed to any region of the mRNA can cause conformational changes, which interfere with protein translation. Evidence for specific translation arrest has been provided by several studies showing that antisense oligos block synthesis of a single protein without reducing the level of the correspondent mRNA.

2. Initiation of protein translation is inhibited when the oligo is targeted to the promoter region or around the initiation codon. It has been shown experimentally that

the constitution of the ribosomal complex and initiation of protein translation may be inhibited by specific antisense oligonucleotides.

3. Before translation takes place in the cytoplasm, the RNA is processed in the nucleus by splicing into its final bioactive form. Antisense oligos directed to the splice junctions of the pre-mRNA interfere with mRNA maturation.

4. In addition to the above mentioned mechanisms, the enzyme RNase H has been described to cleave the mRNA strand while leaving the DNA oligo intact. RNase H recognises the duplex of the oligo and the mRNA and becomes a substrate for the enzyme RNase H. However, the significance of RNase H for the mediation of the antisense effect has not yet been fully elucidated, and therefore is currently not as commonly used as the other three.

The antisense oligonucleotides used in this thesis are directed against a sequence in the mRNA for the $\alpha 2$ integrin subunit. The exact sequence is not available, as the oligonucleotides were purchased ready-made from Biognostik (Germany), and therefore the sequence is confidential.

Method of antisense delivery into fibroblasts

Antisense oligonucleotides were delivered to human dermal fibroblasts as naked DNA. The use of vectors such as lipofectin (Gibco BRL, UK) caused huge decreases in cell viability, and so oligos were delivered successfully and with no cell toxicity in media alone. This delivery was confirmed and quantitated using a time-course delivery of FITC-tagged oligos prior to the start of experiments.

LDH assay to determine toxicity of antisense olgionucleotides

The LDH assay kit (Amersham, Bucks, UK) was utilised in all LDH assessments. The substrate was made up in 12mls of assay buffer and shaken well. 50μl of media from each antisense or serum control–treated well were removed and placed into one well of a 96-well plate. Samples were taken at 0, 8, 16, 24, 48 and 72 hours following antisense/serum application. In the dark, 50μl of substrate mix were added to each well, and the plate immediately covered in foil and left for 30 minutes at room temperature. Still in the dark, 50μl of stop solution (1M Acetic Acid) was added to each well. Each well was checked for bubbles, and any bubbles were removed with a syringe. Plates were then read in a 96-well plate reader at 492nm with no shaking.

Antisense treatment and protein quantitation

Cultures of human dermal fibroblasts were set-up in Flexplates and grown to confluence in 10% FCS as described previously. Once at confluence, cells were pre-incubated in serum-free media containing proline (0.2mM), ascorbate (50mg/ml) and BSA (1mg/ml) for 24 hours. Media was then removed, and replaced with an identical media containing antisense to α2 integrin at a concentration of 2μM (as determined by concentration dose-response curves). The antisense was left on the cultures for 16 hours. Following this, media was changed to contain 10% FCS, proline and ascorbate as above and cells were left in the incubator or mechanically loaded on the Flexercell Strain Unit as described previously for up to 72 hours.

At the end of the incubation period, cultures were trypsinised using a solution of trypsin/EDTA for 2 minutes at 37^0C. Trypisinisation was neutralised using 2 mls of DMEM, and the suspension of cells from 3 wells were pooled and centrifuged at 1500rpm for 5 minutes. The cell pellet was resuspended twice in 2mls of cold PBS and re-centrifuged. Once washed, the pellet was suspended in 2mls of a solution of 1% Biotin (Pierce, UK) in PBS and left for 1 hour at 4^0C with occasional agitation. Following biotinylation, the solution was centrifuged and washed in PBS as before. Cells were then suspended in 0.5ml of RIPA lysis buffer containing 1% SDS/1% NP-40 and a cocktail of protease inhibitors. Samples were left to lyse for 5 minutes at room temperature. Lysed samples were centrifuged at 2500 rpm for 5 minutes to pellet any insoluble protein material. Total protein in the resulting solutions were then quantitated using the Biorad protein assay.

Following total protein quantitation, 300µg of total protein from each sample was aliquoted into an eppendorf. Antibodies to specific integrin subunits (see Antibody section for details) were then added to the lysates at a final concentration of 1:100. Samples were then placed into an eppendorf rotator, and left in a cold room at 4^0C for 3 hours to allow antibody binding. Following this, 10µl per sample of Protein G-Agarose conjugated beads (Affinity Chromatography, San Diego, USA) were added to each eppendorf and once again left rotating at 4^0C for 3 hours to allow antibody-Protein G binding. Immunoprecipitation was terminated following this by centrifuging samples at 2500 rpm for 5 minutes at 4^0C. This centrifugation step precipitated the protein G-antibody complexes. Supernatants were aspirated and discarded, and the pellet washed in cold RIPA lysis buffer (as before) and re-centrifuged twice. The final pellet was then resuspended in 40µl of Tris-Glycine SDS-PAGE sample buffer

(Novex, UK). Pellets were then electrophoresed on a 10% resolving gel and blotted onto nitrocellulose as described previously. Blots were then incubated in streptavidin-HRP conjugated secondary antibody at a final dilution of 1:1000, as described previously for detection of biotinylated proteins. Total and cell-surface levels of integrin $\alpha 2$ were estimated using densitometric scanning in both antisense-treated and non-treated cultures at each time point.

Determination of procollagen synthesis and PCP levels in response to antisense

Experiments were set up as described above, and antisense oligonucleotides added at 2μM to the culture medium. Following 48 hours incubation with or without mechanical load, cultures were processed as described previously for either PCP protein analysis by western blotting, or procollagen synthesis and insoluble collagen deposition analysis by HPLC.

2.2.9 THREE-DIMENSIONAL MECHANICAL LOADING

Loading device

Cells were loaded using the tensioning culture force monitor (t-CFM) designed by Dr Mark Eastwood (Ph.D. Dissertation, University College London/Westminster University). The 3D T-CFM applies uniaxial load to a hydrated collagen gel. (Eastwood, McGrouther and Brown, 1994). The cyclical loading was applied by a force transducer and controlled by a PC. Resultant gels were then both fixed and stained for visualisation by microscopy, or the cells were harvested and subjected to biochemical analysis.

Cells were seeded at a concentration of 1×10^6 cells/ml of Rat tail type I collagen (First Link, UK), in 5 mls of collagen (1mg/ml) and 1.25ml/ml of 10xDMEM. The solution was then neutralised to pH7 using dropwise 5M NaOH and poured into a silicon gel mould. Two plastic mesh bars attached to two aluminium 'A' frames at either end were then coated with a similar solution of collagen and placed at either end of the gel (See Fig. XXX). The entire mould was then placed into the incubator at 37^0C and allowed to polymerise for 20-30 minutes. Once polymerised, the gel was gently released from the edges of the mould using a needle and 25mls of 10% FCS in DMEM was added to the mould on top of the gel. The mould and gel were then placed into the incubator and attached to the force transducer which was in turn connected to the main computer controlling the tensioning activity of the t-CFM. Initial endogenous contraction of the collagen gel was measured by the t-CFM over a course of 8 hours, and the force recorded. Gels were then subjected to 24 and 48 hours of loading, the

load force being an equal and opposite overload programme determined by contraction force (dynes) up to 8 hours. The overload programme applied cyclic mechanical load following contraction as shown in the diagram below(See Fig 2.7). After this time gels were removed carefully and subjected to the appropriate analysis.

Control gel cultures throughout were either free-floating gels, or tethered gels. Both display endogenous tension only, the tethered gel less so as the cells are unable to contract the surrounding collagen. Both were set up in an identical fashion to the loaded collagen gels. Free-floating controls were released from the side of the rubber mould in which the gel has polymerised, and allowed to contract over the experimental time period. Tethered gels were not able to contract as each end is attached to fixed points, thus preventing endogenous tension, but at the same time not applying exogenous force. Control gels were analysed in an identical manner to the loaded gels.

Diagrammatic representations of the system can be found in Figs. 2.5-2.7.

Fig. 2.5: Collagen gel preparation for 3D loading

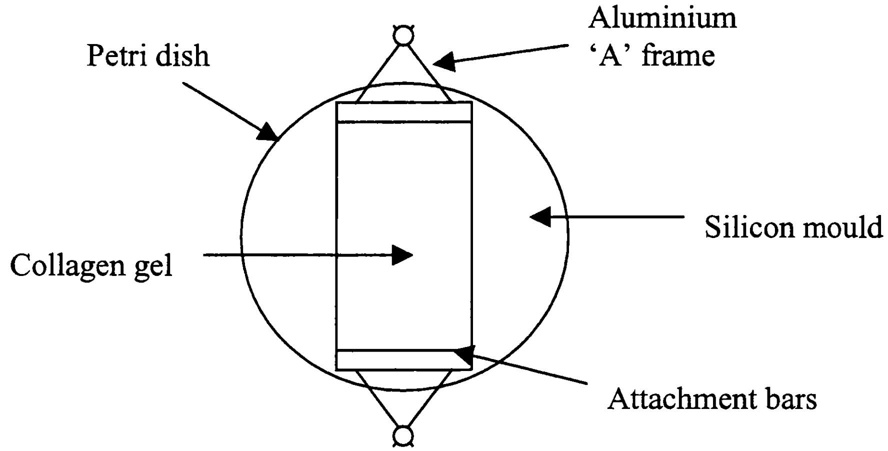


Fig.2.6: The Tensioning Culture Force Monitor:

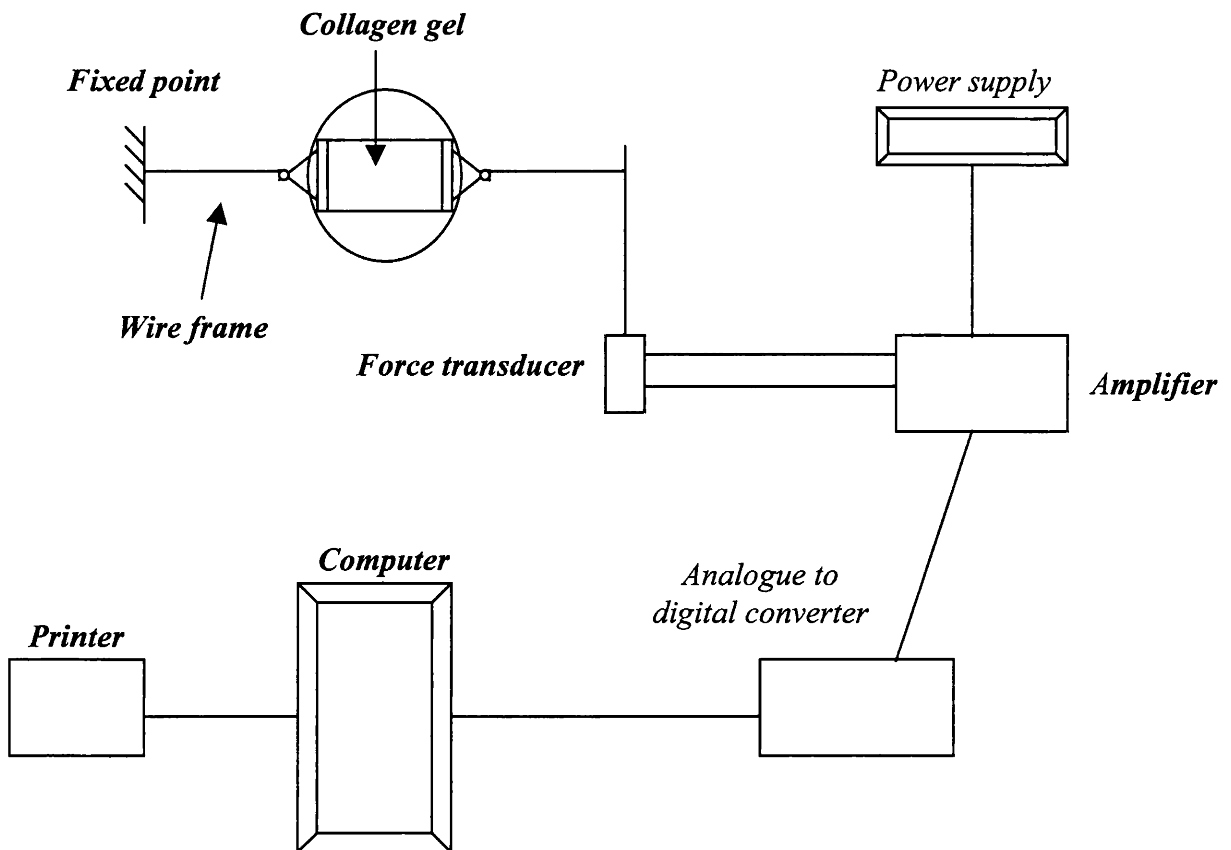
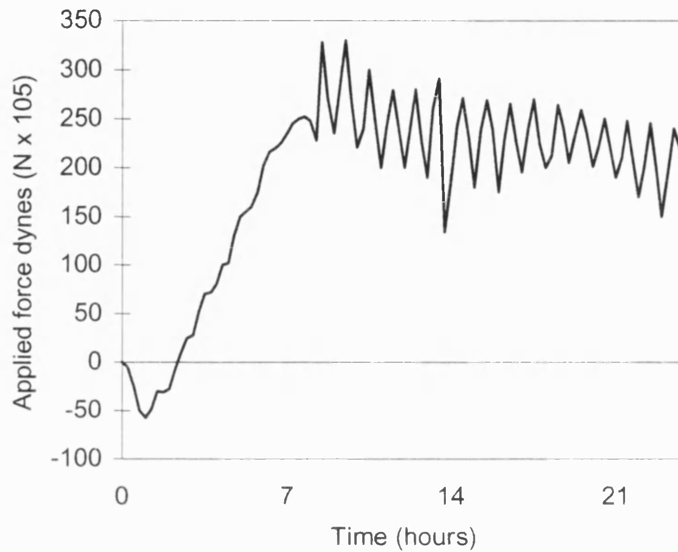


Fig. 2.7: Cyclical overload programme applied on the t-CFM



Figs. 2.5-2.7: The tensioning culture force monitor

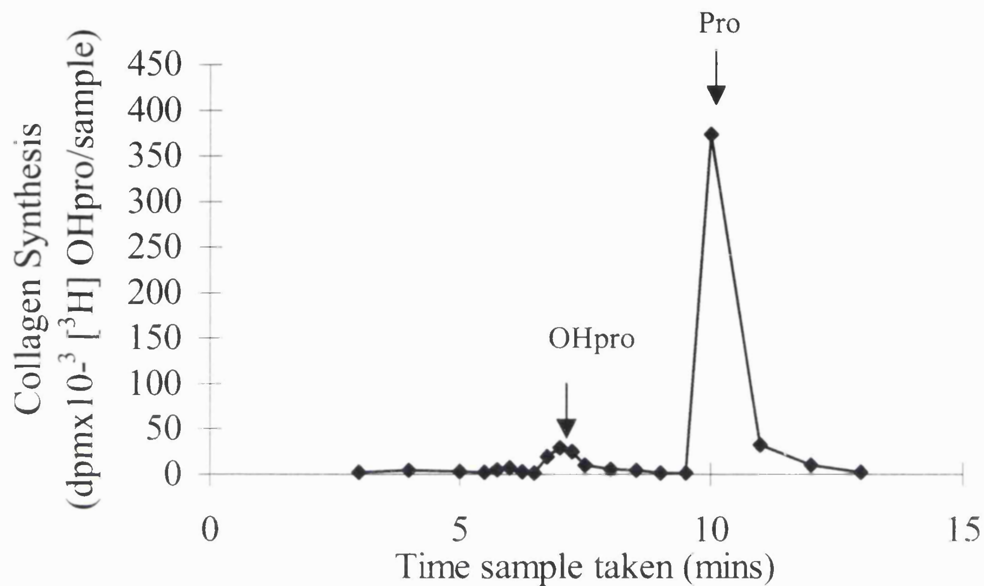
The three figures demonstrate the preparation of collagen gels for loading in the tCFM, the set-up of the tCFM system, and the cyclical overload regimen used throughout the course of this thesis.

2.2.10 Procollagen synthesis assessment in the 3D culture system:

Gels for procollagen synthesis assays were cyclically loaded on the tCFM in the presence of ^{3}H proline (5μCi/ml), which was added with cold proline (0.2μM) into the culture medium along with 10% FCS and ascorbic acid as before. Media samples were removed and precipitated overnight in absolute ethanol to a final concentration of 67% at $4^{0}C$. The samples were then filtered, separating the free hydroxyproline and the intact procollagen and collagen. The collagen gel, which may also contain newly synthesized collagen, was also prepared for analysis. The gel was washed thoroughly 3 times with 67% ethanol overnight at $4^{0}C$, and spun in a Sorvall centrifuge at 15000 rpm for 30 mins. The supernatants were aspirated and discarded, and the pellet was hydrolysed in 6M HCl for 16 hours, as were the precipitated and filtered protein residues from the media samples.

Samples were charcoal filtered following hydrolysis, and the cleared hydrolysate was derivatised for HPLC analysis. Fractions were collected following analysis on a column at regular intervals throughout the run, and the fractions were then counted in a ß-scintillation counter. OHpro peaks were identified by running a OHpro standard (Sigma, as above) first and calculating the area and timing of the peak on the HPLC trace as shown in Figure 2.8

Fig. 2.8: HPLC fractionation of radioactive hydroxyproline



The figure shows a representative trace of a sample of hydrolysed collagen gel containing human dermal fibroblasts following mechanical load in the presence of tritiated proline. Fractions were taken from the HPLC column at the time points shown up to 13 minutes. The peak at around 6.5 minutes is hydroxyproline, as determined by cold amino acid standards, and the peak at 10 minutes is that of unincorporated tritiated proline.

Fig. 2.9: Principal steps involved in quantitation of procollagen synthesis by cells in a collagen gel.

Collagen gel containing cells incubated in media

containing serum and 5μCi/ml ^{3}H proline

↓

Following treatment, gels and media separated

↙ ↘

Media samples precipitated in

centrifuged

67% ethanol at 4^0C overnight

Gel samples washed and

in 67% ethanol

↓

Media and gel samples hydrolysed in 6M HCl for 16 hours.

↓

Resultant samples derivatised in 4-NBD-Cl and borate buffer

and run on a reverse-phase HPLC column

↓

Fractions collected from column every 15 seconds between minutes 3 and 13 of

the HPLC run

↓

Fractions mixed with scintillation fluid and analysed for

radioactive OHpro content using a ß-scintillation counter

↓

Newly synthesised procollagen calculated by analysing area

under radioactive OHpro peak on resultant trace.

2.2.11 ***Inhibitors of procollagen synthesis in collagen gels***

The collagen synthesis inhibitors used in these studies were ethyl 3,4 dihydroxybenzoate (a prolyl-4-hydroxylase inhibitor) and 3,4 dehydro-DL-proline (proline analogue) and were both obtained from Sigma Ltd (UK). Ethyl 3,4 dihydroxybenzoate (EDHB) and 3,4 dehydro-DL-proline (DHP) were both used within concentration ranges previously shown to inhibit procollagen synthesis in different cell types (Rocnik et al, 1998). EDHB was used at 400μM and DHP at 1mM. These compounds have also previously been shown to affect collagen synthesis without affecting mesenchymal cell/ECM attachment, α-smooth muscle actin expression, total protein or cellular ATP levels (Rocnik et al, 1998). Compounds were added to the culture media following an 8 hour contraction period, and immediately prior to the onset of loading.

2.2.12 ***Determination of proliferation of cells within a collagen gel***

Gel and media were separated following individual treatments. Gels were then cleaned briefly in PBS twice and incubated in a solution of trypsin/EDTA, 2mg/ml collagenase (Type I, Sigma Chemicals Ltd , as before) in 50mM Tris/10mM Calcium acetate for 1 hour at 37^0C. Samples were then pipetted a number of times through a fine needle to separate cells and digested protein. Aliquots of digested sample were then taken and mixed with a solution of trypan blue to determine cell number and

viability. 10μl of this aliquot was then placed into a hemocytometer and viewed down a light microscope to assess number of cells within a calculated fraction of the total gel. Total cell number was then calculated from this.

2.2.13 Scanning Electron Microscopy of Collagen Gels:

Gels were set up as described previously and loaded for 24 hours alongside a rigid control. Control gels containing no cells were also set up and treated in an identical manner. After 24 hours, the media was removed and the gels were fixed in 25 mls of 2.5% gluteraldehyde in phosphate buffer. Gels were then left to fix overnight at 4^0C. Samples were then biologically fixed again in a solution of Osmium Tetroxide, washed in phosphate buffer and freeze-dried in liquid nitrogen to achieve the desired section and orientation of the gel for analysis.

Resulting samples were then dehydrated through a series of alcohol concentrations (50%-100%) for 30 minutes each. Samples were then point dried by soaking in HMDS (Hexamethyl-di-silazane) for 30 minutes followed by air-drying. Pieces of each gel were then mounted onto aluminium stubs, coated with gold for 3 minutes and analysed under SEM.

2.2.14 Statistical analysis

All data are presented as mean ± standard errors of the mean (SEM). Statistical analysis was performed using an unpaired students t-test for single group comparisons and Newman-Keuls one way analysis of variance (ANOVA) for multiple group comparisons. All analysis comparing mechanically loaded samples with control samples was conducted using a Students' t-test. Average values of replication or collagen synthesis were calculated (n+6) for all groups in each experiment. Westerns and northern analysis were all conducted in parallel 3 times for each set of experiments to gain an average from densitometric scanning. Differences were considered statistically significant at $P<0.05$, $p<0.01$ or $p<0.001$.

CHAPTER THREE

Results:

Regulation of procollagen synthesis

3.1 INTRODUCTION

Procollagen synthesis and degradation are complex and tightly regulated processes, the basis of which is described in Chapter 1.3. The fibroblast is the primary cell involved in the synthesis of collagen and other extracellular matrix molecules in skin *in vivo*. Therefore, understanding the regulation of this process is vital to unravelling mechanisms of excessive collagen deposition in pathological situations such as dermal scarring. As alluded to in the general introduction, mechanical load may prove to be an important regulator of procollagen metabloism.

Mechanical load has been proven to be stimulatory in many different cell types *in vitro*. One such example is in the stimulation of new matrix synthesis by mesenchymal and endothelial cells. Studies addressing the effects of load use *in vitro* systems, which apply a static or cyclical force to the cultures. Cyclical mechanical load may be applied to cells, as it is believed that the constant movement of the cultures provides a closer representation to an *in vivo* environment where cells are constantly exposed to changes in tension. The stimulation of collagen synthesis in response to cyclical exogenous load has been reported in different cardiac fibroblast cell lines previously (Carver *et al*, 1991; Butt and Bishop, 1997, Sumpio *et al*, 1995) and in lung fibroblasts (Bishop *et al*, 1995), but never before in dermal fibroblasts of any species.

The effects of load on collagen degradation have been less well studied. Basal or intracellular degradation of collagen has previously been shown to be increased at the same rate as procollagen synthesis in the presence of mechanical load in cardiac

fibroblasts (Butt R. P., Ph.D. Thesis, University of London, 1996). However, extracellular degradation of collagen by matrix metalloproteinses (MMP's) is increased in some models of mechanical load. A recent study by Lee and colleagues showed increased MMP2 and MMP9 levels in mechanically loaded smooth muscle cell cultures, and this was directly related to the degradation of collagen within the cultures (Lee *et al*, 1998). The overall net effects of mechanical load on procollagen metabolism are still being investigated.

The involvement of growth factors in the cellular response to mechanical load is one of controversy within the field. A number of studies have shown that mechanical load can stimulate collagen synthesis above basal levels in the absence of any growth factors, but that this effect can be further enhanced in the presence of growth factors or low levels of serum (Carver *et al*, 1991). Others have reported that growth factors are required in order to see any response to load in terms of collagen synthesis (Butt and Bishop, 1997). The nature of this form of synergy between load and growth factors is still poorly understood.

The serine protease thrombin and the growth factor TGFβ both represent factors that are present at the early and late stages of dermal wound healing (For review see Clarke, 1997). Both factors have also been shown to stimulate fibroblast procollagen synthesis both at the transcription and protein levels (Roberts *et al*, 1985; Chambers *et al*, 1997). As mentioned, synergy between growth factors such as these and mechanical forces have been described previously in mesenchymal cells, and this synergy has been implicated in the development of fibrotic conditions such as cardiac hypertrophy (Bishop *et al*, 1994). However, the effects of growth factors in conjunction with the application of mechanical load have not been previously

investigated in dermal fibroblasts. The possible synergy between these two factors must be assessed *in vitro* in dermal fibroblasts in order to further an understanding of *in vivo* cellular responses to forces during dermal repair.

Aims

The aim of this chapter is to assess the effect of serum, TGFß and thrombin on the stimulation of procollagen synthesis and degradation in primary human dermal fibroblasts. The effects of 2D cyclical mechanical load on procollagen synthesis in the presence of these growth factors was then assessed to ascertain if the dermal fibroblasts require selected growth factors to respond to load, or act synergistically. It has been reported previously that all these factors have been previously published to stimulate procollagen synthesis in a variety of cell types and *in vivo* fibroses, and therefore provide a valuable basis for the study of effects of load on **enhanced** collagen synthesis. The effects of load on non-collagen protein synthesis are also investigated to determine if load specifically affects collagen synthesis or whether it has a general effect on protein metabolism.

3.2 RESULTS

3.2.1 Procollagen production and degradation in dermal fibroblasts is increased in response to serum, TGFβ and thrombin.

The data in figures 3.1 and 3.2 show that procollagen production and degradation was increased linearly by serum and growth factors above serum-free controls over a time course of 72 hours. Procollagen production was enhanced after 72 hours over serum-free controls by 150±12%, 250±23% and 350±31% in cultures maintained in thrombin, TGFß and 10% FCS respectively, all statistically significant to a level $p<0.001$ as compared to serum-free controls. Degradation of procollagen also increased over the 72 hours culture period in identical cultures by 100±21% ($p<0.01$), 170±31% ($p<0.001$) and 210±32% ($p<0.001$) respectively above serum-free controls. Total procollagen synthesis in these cultures is represented in Fig. 3.3. Total procollagen synthesis in 10% FCS, TGFß and thrombin treated cultures after 72 hours was 255±26%, 205±18% and 138±12% respectively, all statistically significant to a level $p<0.001$ as compared to serum-free cultures.

Fig. 3.4 shows that there was measurable fibroblast replication in the confluent cultures maintained in 0% FCS or TGFß over 72 hours. There were slight increases in 10% FCS and thrombin treated cultures over 72 hours, 25±5% ($p<0.05$) and 18±4% ($p<0.05$) respectively. Such increases were corrected for in all subsequent procollagen metabolism experiments and all values were given as nmol OHpro/$2x10^5$ cells.

Fig. 3.1:

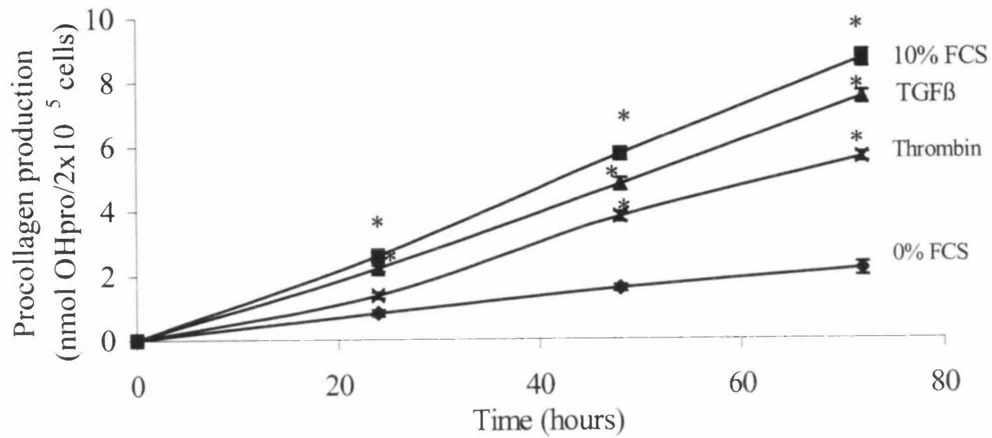


Fig. 3.2:

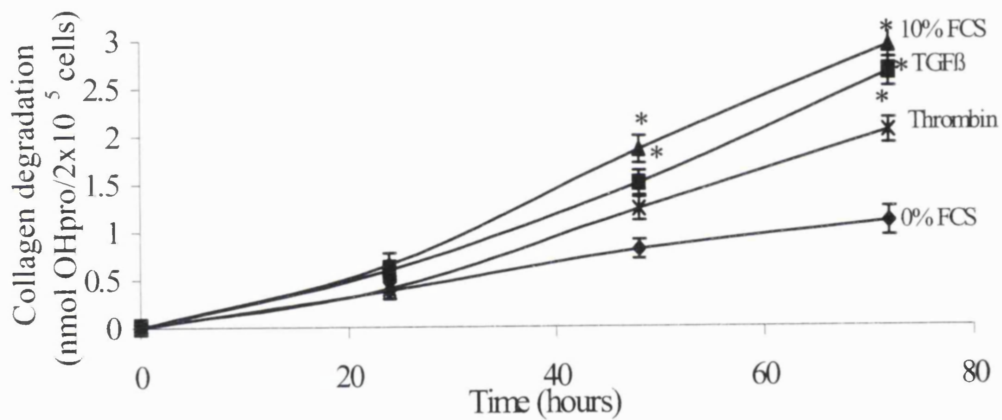


Fig. 3.3:

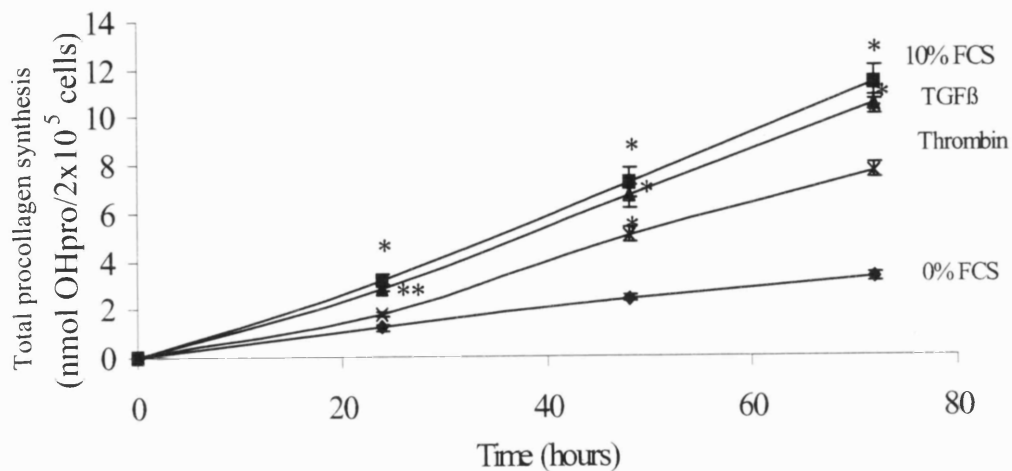
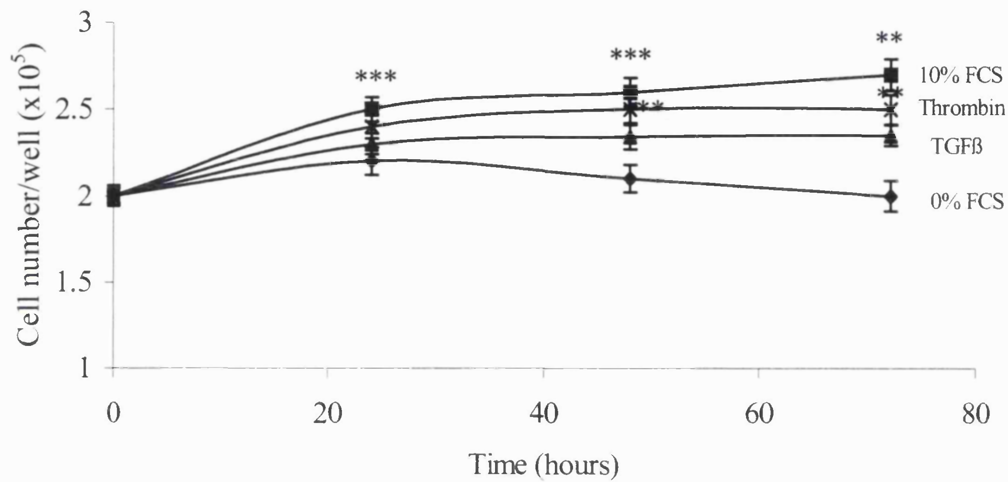


Fig. 3.4:



Figs. 3.1-3.4: Procollagen production and degradation in dermal fibroblasts is increased in response to serum, TGFβ and thrombin.

The effects of 10% FCS, TGFß (10pM) and thrombin (2nM) on procollagen production, degradation and cell number were assessed over a time course of 72 hours. Data is expressed as nmol OHpro/$2x10^5$ cells for procollagen, and cell number as $x10^5$. Total procollagen synthesis is calculated as procollagen production + degradation for each time point. * and ** and *** denote $p<0.001$ and $p<0.01$ and $p<0.05$ level of significance respectively compared to serum free control at that time point.

3.2.2 Mechanical load and serum or growth factors enhance total procollagen synthesis in dermal fibroblasts.

Fig 3.5 shows total procollagen synthesis in fibroblast cultures, i.e. total procollagen production + degradation for each time point. Cultures mechanically loaded in serum-free media did not show any significant change in procollagen synthesis at any time point investigated. Similarly, mechanical load did not stimulate any increase in procollagen synthesis in cultures containing serum, TGFß or thrombin at 24 hours. Mechanical load stimulated total procollagen synthesis in the presence of 10% FCS by 95±4% ($p<0.001$) after 48 hours incubation. TGFß also elicited a similar kinetic effect, stimulating a 55±3% ($p<0.001$) increase in procollagen synthesis at 48 hours and then no significant increase in the **rate** of synthesis over unloaded control between 48 and 72 hours. Load in the presence of thrombin also enhanced total procollagen synthesis over the respective growth factor control at 48 hours by 40±5% ($p<0.05$).

Figure 3.6 demonstrates that mechanical load did not enhance procollagen production above non-loaded control cultures in cells maintained in serum-free media over 72 hours. However, cells mechanically loaded in the presence of 10% FCS exhibited a 55±4% ($p<0.001$) increase in procollagen production over serum control after 48 hours. The 10% FCS control culture continued to produce procollagen at the same rate during the 48-72 hour period as during the 0-48 hour period. However, mechanical load had no significant effect on procollagen production compared with control in the 48-72 hour period. Fig 3.6 shows there was no significant difference in procollagen production between either of the growth factors controls and the loaded cultures after 24 hours incubation. At 48 hours however, TGFß and thrombin-treated

cultures subjected to load demonstrated enhanced procollagen production 45±3% ($p<0.001$) and 38±4% ($p<0.01$) respectively above controls. Production in both the loaded cultures decreased over the 48-72 hour period, over this period there was no significant increase in the rate of production compared to growth factors controls.

Fig. 3.7 shows degradation of procollagen in response to mechanical load and serum or growth factors. The effect on procollagen degradation was the same in loaded and control cultures as shown for production. As with procollagen production, degradation increased in loaded samples compared with unloaded during the 24-48 hour period. The rate of degradation began to decrease to control levels in all cultures during the 48-72 hour period.

Fig. 3.8 shows the effect of mechanical load and growth factors on human dermal fibroblast proliferation over 72 hours. Data indicates that cells growth factors and serum stimulated cell proliferation from 24 hours up to the 72 hours time point compared to serum-free controls, as shown previously in Fig. 3.4. However, the application of mechanical load did not stimulate any further significant increase in cell proliferation in the presence of serum, TGFß or thrombin at any time point.

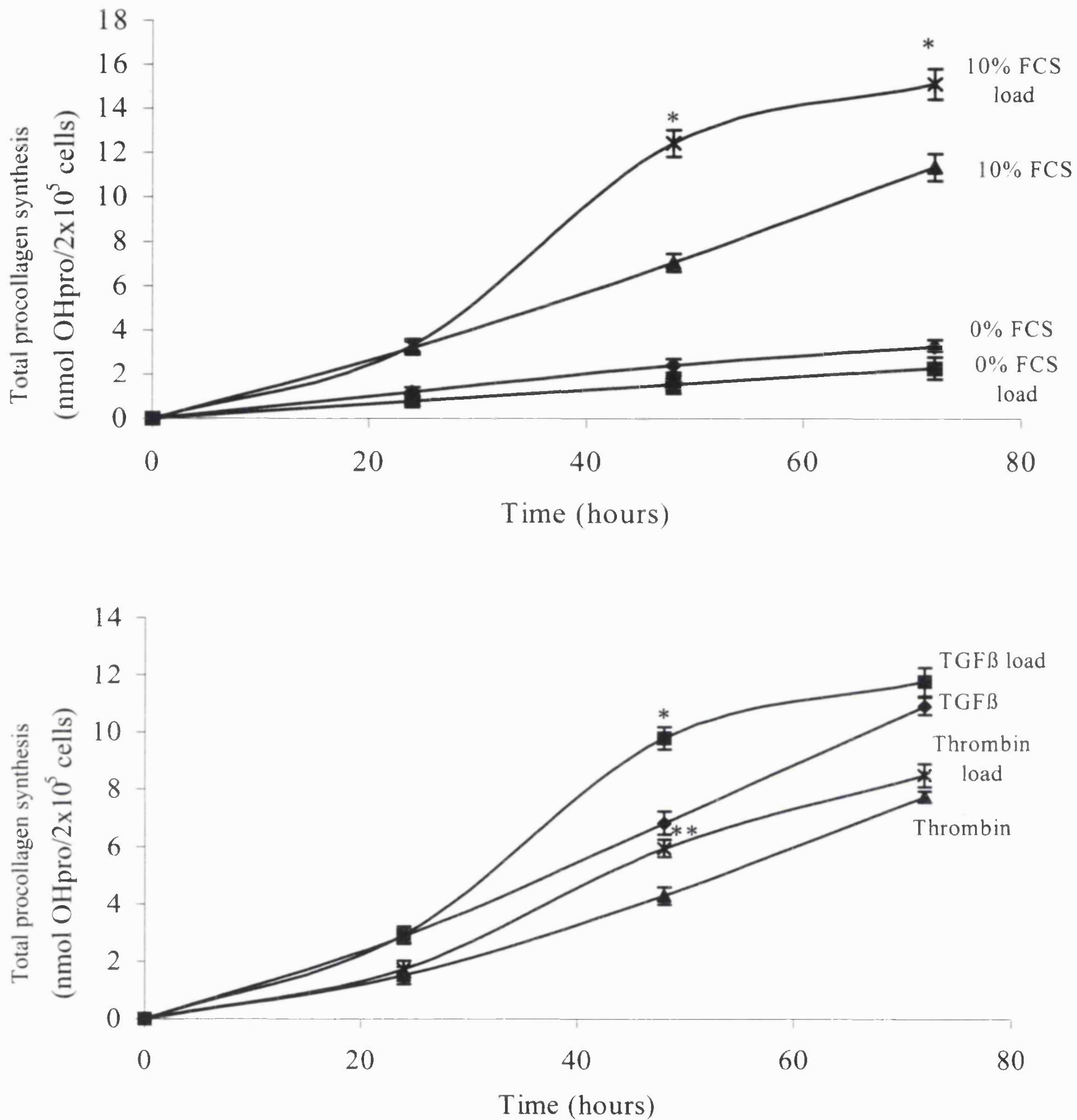


Fig. 3.5 Mechanical load and serum or growth factors enhances total procollagen synthesis in dermal fibroblasts.

The effects of 10% FCS (top panel), TGFß (10pM) and thrombin (2nM) (bottom panel) in the presence of mechanical load on total procollagen synthesis were assessed over a time course of 72 hours. Data is expressed as nmol OHpro/10^5 cells. Total procollagen synthesis is calculated as procollagen production + degradation for each time point. * and **denote $p<0.001$ and $p<0.01$ level of significance respectively compared to growth factor control at that time point.

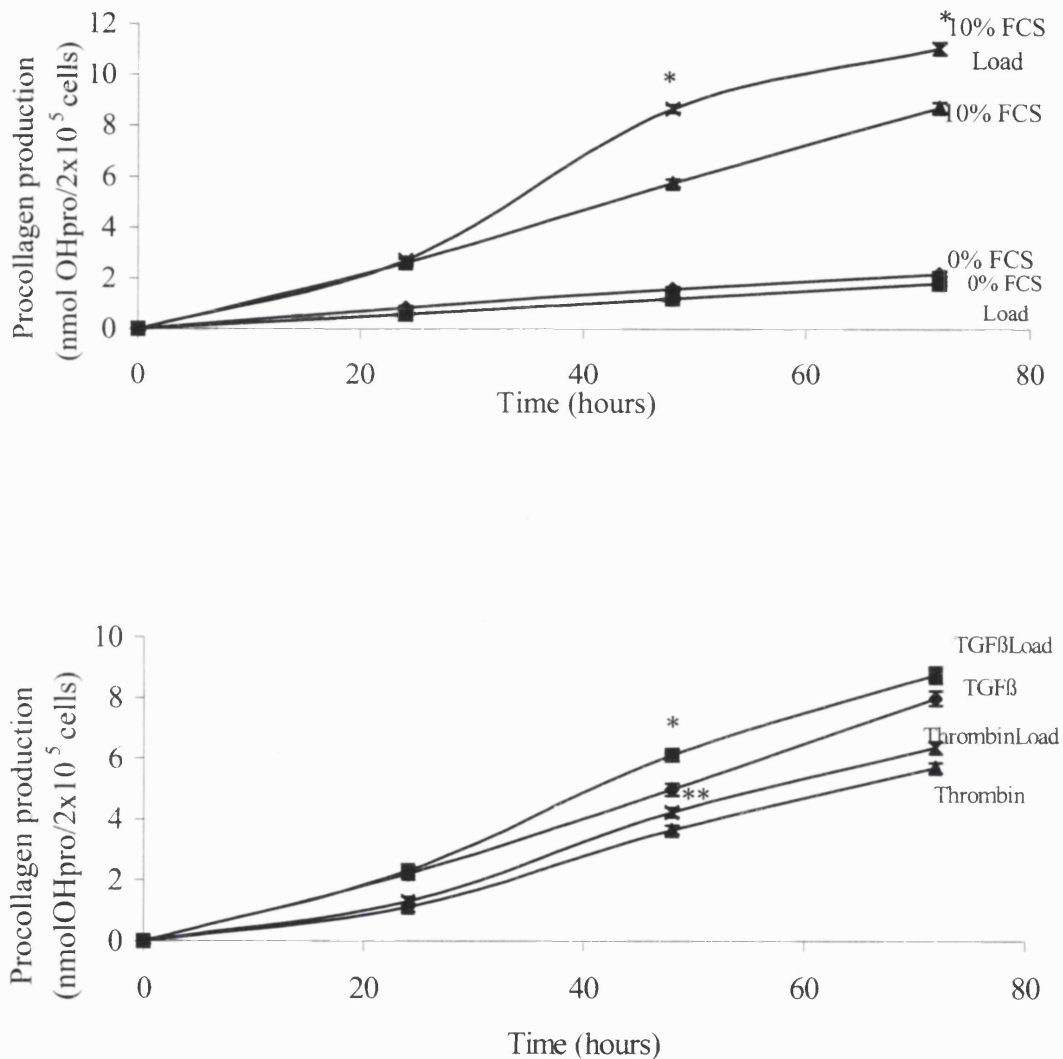


Fig. 3.6: Mechanical load and serum or growth factors enhances procollagen production in dermal fibroblasts.

The effects of 10% FCS (top panel), TGFß (10pM) and thrombin (2nM) (bottom panel) in the presence of mechanical load on procollagen production were assessed over a time course of 72 hours. Data is expressed as nmol OHpro/10^5 cells. * and **denote $p<0.001$ and $p<0.01$ level of significance respectively compared to growth factor control at that time point.

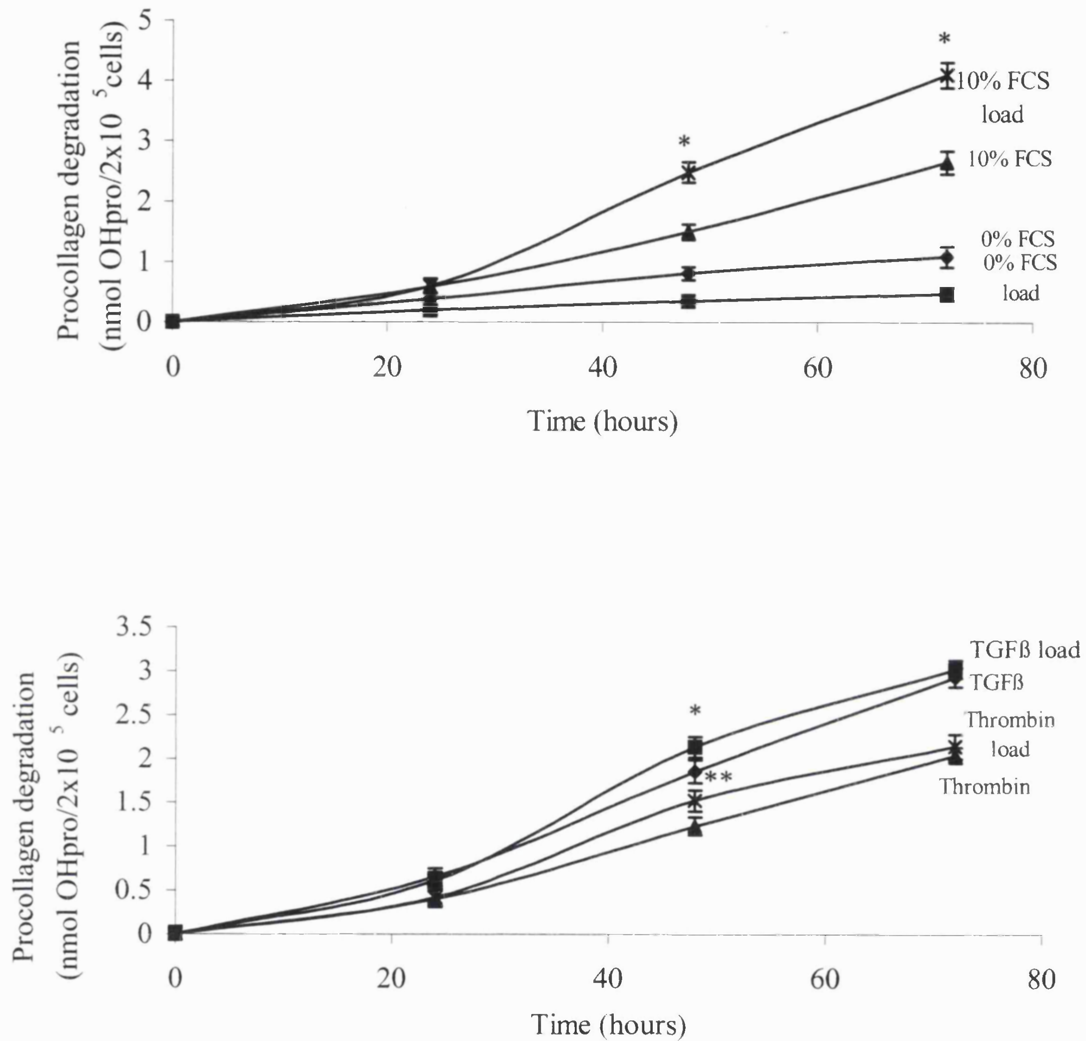


Fig. 3.7 Mechanical load and serum or growth factors enhances procollagen degradation in dermal fibroblasts.

The effects of 10% FCS (top panel), TGFß (10pM) and thrombin (2nM) (bottom panel) in the presence of mechanical load on procollagen degradation were assessed over a time course of 72 hours. Data is expressed as nmol OHpro/10^5 cells. * and **denote $p<0.001$ and $p<0.01$ levels of significance respectively compared to serum free control at that time point.

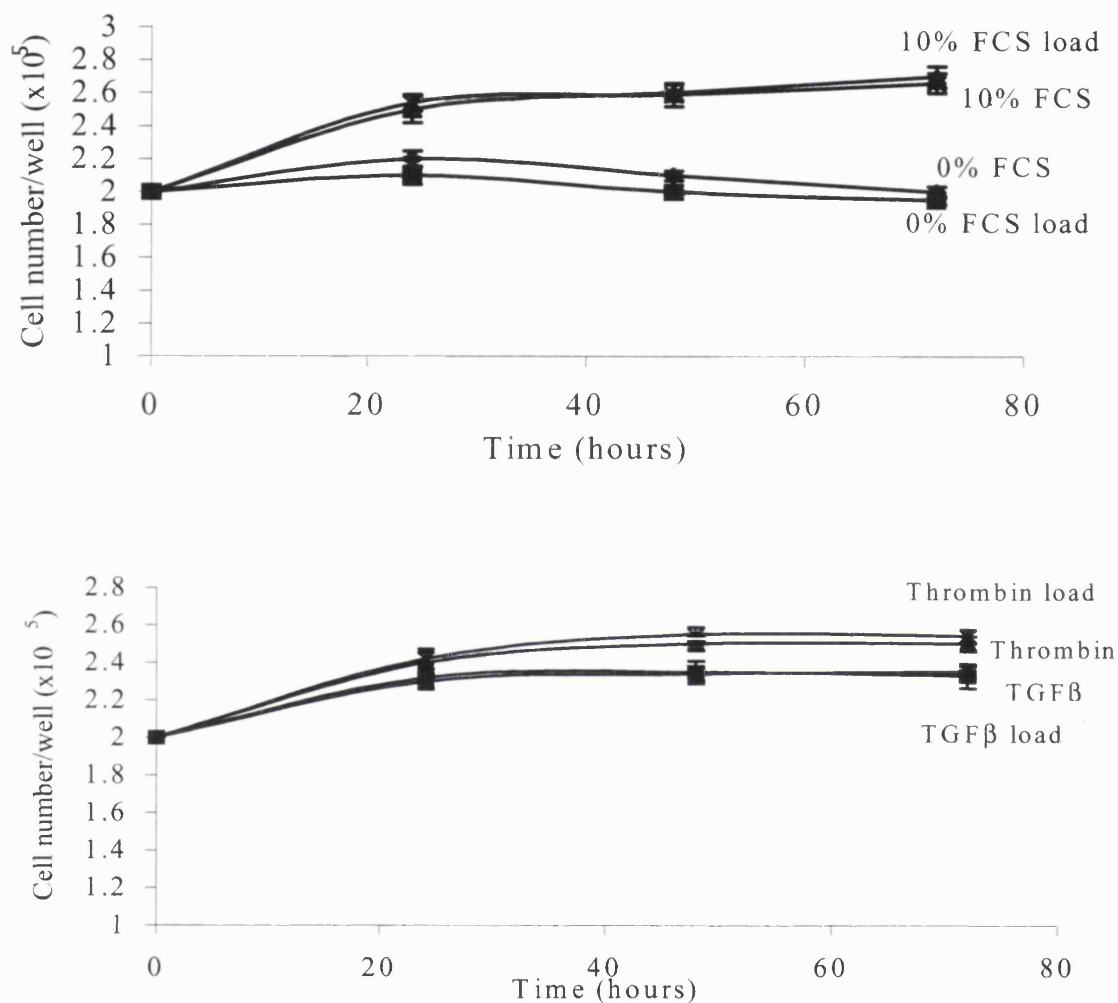


Fig. 3.8: Mechanical load with serum or growth factors has no effect on dermal fibroblast proliferation.

The effects of 10% FCS (top panel), TGFß (10pM) and thrombin (2nM) (bottom panel) in the presence of mechanical load on cell number were assessed over a time course of 72 hours. Data is expressed as no. of cells/well ($x10^5$).

3.2.3 Reproducibility of procollagen synthesis measurements in cultures treated for 48 hours with mechanical load in the presence of 10% serum, TGFβ and thrombin.

The data in table 3.1 indicate the reproducibility of the procollagen synthesis measurements in response to growth factors and load. Cultures were incubated for 48 hours in the presence of 10% serum, TGFß or thrombin in the presence or absence of mechanical load.

The range of increase in procollagen synthesis in the presence of mechanical load and 10% FCS was between 40±4% and 98±4% above serum control level (A). The range of increase in procollagen synthesis for cells incubated with TGFß in the presence of load was between 24±3% and 56±5% over unloaded control (B). The responses of cells to load and thrombin ranged between 22±3% and 54±4% increase in procollagen synthesis as compared to growth factor control (C).

A} 0% FCS:

Experiment	Procollagen production		Procollagen degradation		**Procollagen synthesis**	
	Control	Load	Control	Load	**Control**	**Load**
1	1.6±0.2	1.2±0.1	0.8±0.1	0.3±0.1	**2.4±0.3**	**1.5±0.4**
2	2.6±0.4	2.4±0.3	1.2±0.2	0.8±0.2	**3.8±0.4**	**3.2±0.4**
3	3.1±0.3	2.7±0.3	1.3±0.3	1.0±0.2	**4.4±0.5**	**3.7±0.4**

B} 10% FCS:

Experiment	Procollagen production		Procollagen degradation		**Procollagen synthesis**	
	Control	Load	Control	Load	**Control**	**Load**
1	5.7±0.3	7.9±0.2*	1.5±0.1	2.4±0.2	**7.2±0.4**	**10.4±0.4***
2	6.4±0.2	9.3±0.3*	1.7±0.2	3.0±0.2	**8.1±0.4**	**12.3±0.5***
3	8.1±0.3	11.0±0.4*	2.4±0.1	4.4±0.3	**10.5±0.4**	**15.4±0.7***

C} TGFß:

Experiment	Procollagen production		Procollagen degradation		**Procollagen synthesis**	
	Control	Load	Control	Load	**Control**	**Load**
1	4.9±0.1	6.1±0.2*	1.8±0.2	2.2±0.1	**6.7±0.3**	**8.3±0.3***
2	5.4±0.2	8.1±0.2*	1.0±0.1	2.2±0.2	**6.4±0.3**	**10.3±0.4***
3	7.0±0.3	9.4±0.3*	1.4±0.1	3.6±0.3	**8.4±0.4**	**13.0±0.6***

D} Thrombin:

Experiment	Procollagen production		Procollagen degradation		**Procollagen synthesis**	
	Control	Load	Control	Load	**Control**	**Load**
1	3.6±0.2	4.2±0.2***	1.2±0.1	1.5±0.1	**4.8±0.3**	**5.7±0.3****
2	4.1±0.2	5.8±0.2***	1.3±0.1	2.0±0.2	**5.4±0.3**	**7.8±0.4****
3	5.7±0.2	6.9±0.3***	2.2±0.1	2.9±0.3	**7.9±0.3**	**9.8±0.6****

Table 3.1: Tables showing reproducibility of procollagen synthesis measurements for cultures treated for 48 hours with mechanical load in the presence of 0% FCS (A), 10% serum (B), TGFß (C) and thrombin(D).

Values are shown as nmol OHpro/well±SEM over a 48 hour incubation period. All values were corrected for any change in cell number, throughout the course of the experiment. * denotes p<0.001, ** denotes p<0.01and *** denotes p<0.05 compared to respective growth factor controls

3.2.4 Mechanical load and growth factors increases procollagen α1(I) mRNA after 48 hours.

The Northern analysis of procollagen α1(I) mRNA in Fig 3.9 demonstrated that the mRNA is upregulated in response to load and 10% FCS, TGFß and thrombin after 48 hours of treatment compared to unloaded controls. These data show that the increased mRNA expression is only seen at 48 hours, as demonstrated with procollagen protein (see Fig. 3.5). Densitometric scanning of northern blots from individual experiments showed that 10% FCS and load stimulated a 125±18% ($p<0.001$) increase in procollagen α1(I) mRNA levels over unloaded serum control after 48 hours treatment.

TGFß and load stimulated a 105±22% ($p<0.001$) increase, and thrombin a 45±12% ($p<0.05$) increase in mRNA over growth factor controls, both after 48 hours incubation. Serum-free samples showed no increase at any time point in procollagen α1(I) mRNA in response to load, which reflects the procollagen protein data seen in Fig. 3.5.

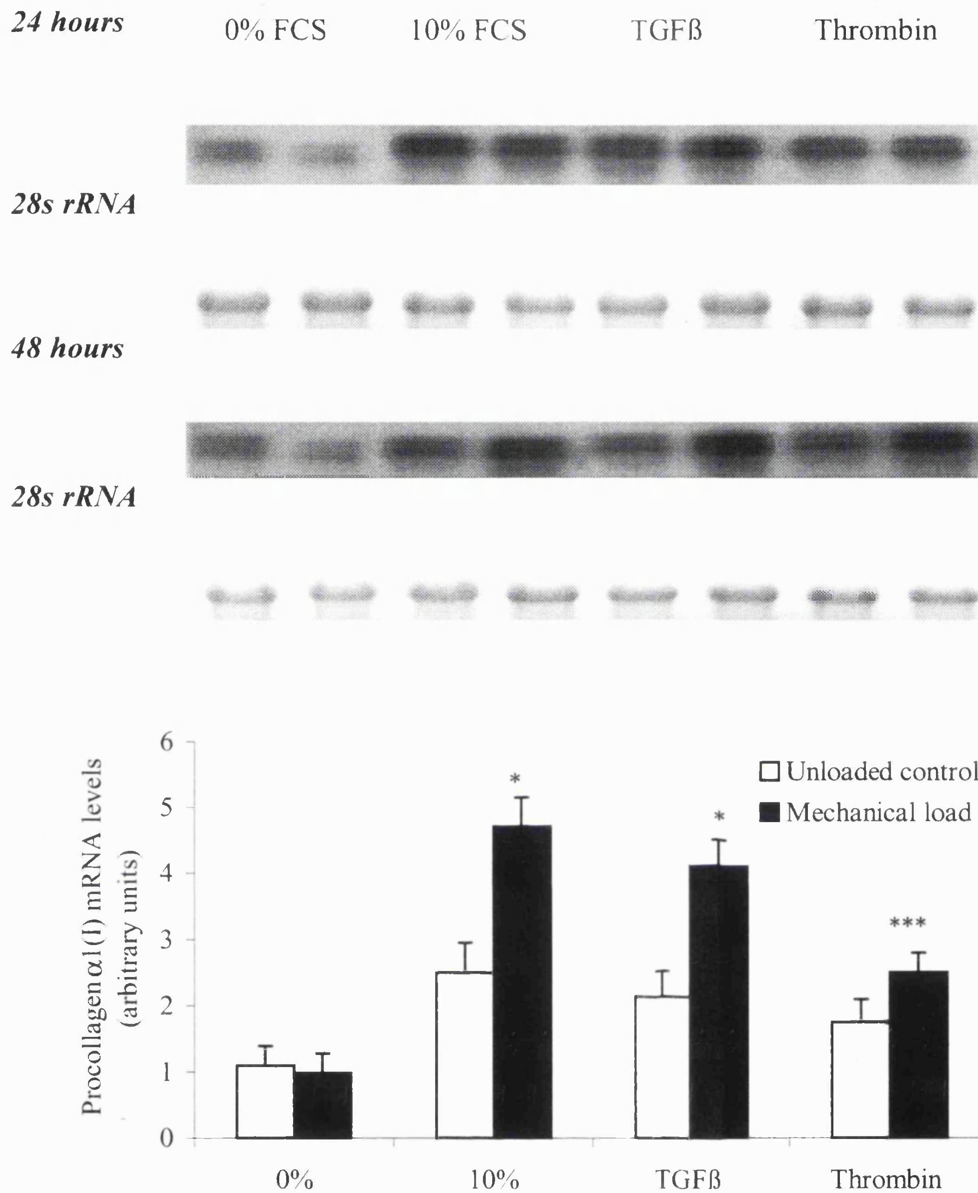


Fig. 3.9: Mechanical load and growth factors increases procollagen α1(I) mRNA after 48 hours

Northern analysis showing representative blots of procollagen α1(I) mRNA levels after 24 and 48 hours in the presence of load and growth factors. 28s rRNA is shown as a loading control. Quantitation of mRNA in samples at 48 hours using densitometric scanning is shown in the bottom graph. * and *** denotes $p<0.001$ and $p<0.05$ compared to unloaded respective control. Experiments were repeated three times for each condition and the SEM of these data is shown.

3.2.5 Conditioned medium from stretched cells does not stimulate procollagen synthesis in new cultures

Table 3.2 shows data indicating that conditioned medium from mechanically loaded cells does not enhance new procollagen synthesis in fresh cultures of human dermal fibroblasts. As previously shown in fig. 3.5, 10% FCS stimulated collagen synthesis in dermal fibroblasts after 48 hours by 140% ($p<0.001$) above serum-free control. There was no significant difference between respective control cultures and cultures treated with conditioned media from cells loaded for 24 or 48 hours.

	0% FCS			10% FCS		
	Control	24h conditioned medium	48h conditioned medium	Control	24h conditioned medium	48h conditioned medium
Total procollagen synthesis (nmol OHpro)	**4±0.3**	**4.2±0.5**	**3.4±0.2**	**9.5±1.0§**	**9±1.2§**	**8.6±0.7§**

Table 3.2: Conditioned media from stretched cells does not stimulate procollagen synthesis in new cultures

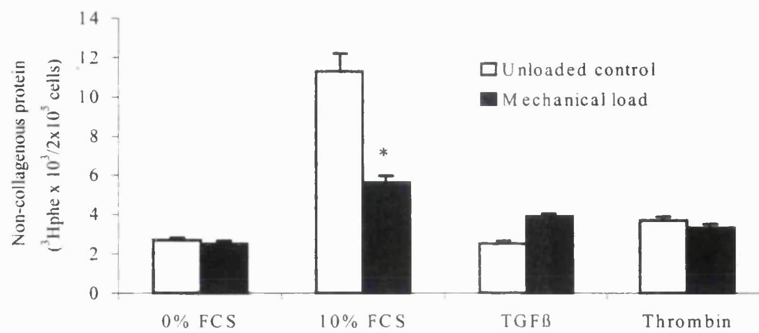
Cells were mechanically loaded in the presence of either 0% or 10% FCS for 24 or 48 hours. The media from these cultures was then removed and placed directly onto fresh cultures of fibroblasts. Total procollagen synthesis was then measured after 48 hours incubation with the conditioned media. Results are expressed in nmol OHpro/ $2x10^5$ cells following subtraction of the time 0 value to control for procollagen transferred in media from loaded cell cultures. "Control" columns indicate the amount of procollagen synthesis measured in cultures following 48 hours incubation with fresh 0% or 10% serum respectively. Experiments were repeated twice for each time point. § denotes $p<0.001$ level of significance compared to serum alone control equivalent.

3.2.6 Mechanical load and growth factors do not increase the rate of non-collagenous protein synthesis.

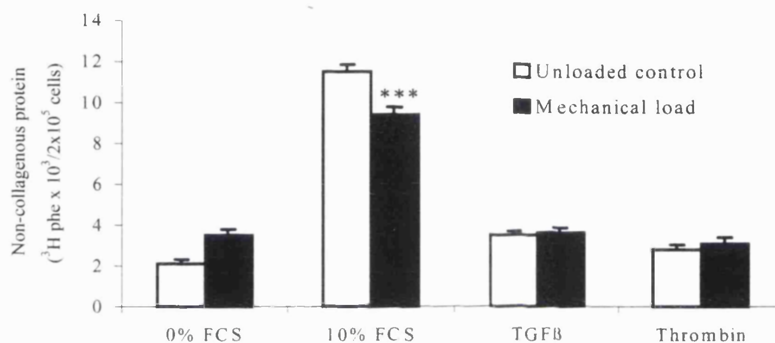
Non-collagenous protein synthesis was assessed in identical cultures to those used for procollagen synthesis measurements. Cells were treated as for procollagen metabolism experiments, but with ^{3}H phenylalanine added into the culture medium. Cultures were then harvested, hydrolysed and fractions counted in a ß-scintillation counter. Cultures treated with 10% FCS exhibited a 340±20%, 450±25% and 420±35% increased rate of total NCP at 24, 48 and 72 hours respectively, compared to respective serum-free controls. TGFß and thrombin did not significantly increase the rate of total non-collagenous protein synthesis at any of the time points investigated.

Figure 3.10 indicates that mechanical load did not increase total cellular non-collagenous protein synthesis in the presence of serum, TGFß or thrombin at any of the time points investigated. However, load did significantly decrease the rate of total non-collagen protein synthesis at both 24 and 48 hours (but not 72 hours) by 55±6% ($p<0.001$) and 22±4% ($p<0.05$) respectively compared to unloaded serum controls.

24 hours



48 hours



72 hours

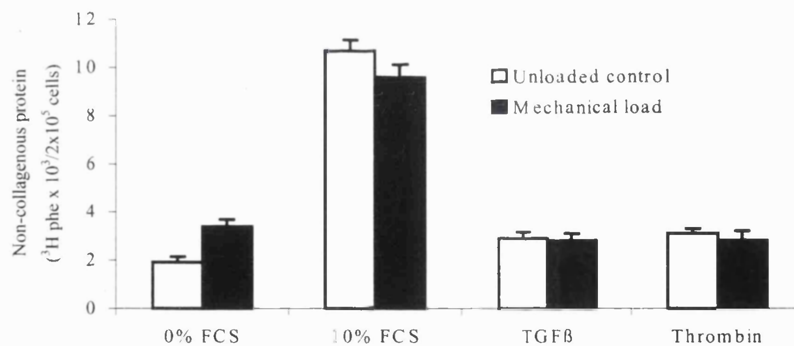


Fig. 3.10: Mechanical load and growth factors do not increase non-collagenous protein synthesis.

Data represents measurements taken at 24, 48 and 72 hours. Values are represented as ^{3}H phenylalanine x 10^{4}/well. All values were corrected for any changes in cell number. * and *** denotes $p<0.001$ and $p<0.05$ as the level of significance between loaded and respective unloaded control.

3.3 DISCUSSION

The aim of this chapter was to characterise the effects of load and growth factors on procollagen synthesis in human dermal fibroblasts. Initially, the effects of the serum and TGFß on procollagen synthesis in the primary cultures of human dermal fibroblasts were investigated. Both serum and TGFß stimulated procollagen synthesis by approximately 100-120% compared to serum-free control cultures after 24 hours of incubation, and this was increased after 48 hours. Procollagen/collagen degradation products were also assessed in the same cultures by measuring free hydroxyproline in the supernatants of the samples. The level of total procollagen degraded was not significantly altered in loaded versus control samples, suggesting that the effects of load in this system are in promoting net procollagen synthesis.

The effects of TGFß and serum on procollagen synthesis have been previously reported in human dermal fibroblasts (Montesano *et al*, 1984). The effects of thrombin on dermal fibroblast collagen production has not been previously reported, but thrombin has been shown to be a potent mitogen and stimulator of procollagen production in smooth muscle cells and fetal lung fibroblasts (Dabbagh *et al*, 1998). Increased fibroblast procollagen synthesis in response to TGFß and thrombin have previously been shown *in vitro* to be mediated primarily via the TGFß receptors (Ferguson et al, 1993) and protease activated receptor-1 (PAR-1) (Chambers et al, 1998) respectively.

Both TGFß and thrombin are present during dermal wound healing *in vivo* following clotting cascade and inflammatory cell recruitment. In particular, TGFßs 1-3 have

been shown to be key players in the formation of dermal scars (Shah *et al*, 1994). Whilst it is currently unproven, it is largely assumed that the same receptors involved in procollagen synthesis in response to thrombin and TGFß *in vitro* are also involved *in vivo*. Current research is addressing these questions using specific inhibitors of both receptors and growth factors in *in vivo* models of fibrosis and scarring.

The procollagen protein data agrees with northern blots indicating upregulation of $\alpha 1(I)$ procollagen gene expression in response to growth factors at 24 and 48 hours. The application of mechanical load enhanced the response to growth factors after 48 hours only. However, when non-collagenous protein synthesis was quantitated, data indicated that although growth factors upregulated non-collagenous protein, mechanical load did not further enhance this effect. This would suggest load is specifically changing levels of new procollagen synthesis within the cell. However, this does not rule out specific fluctuations in other matrix proteins, which may also be specifically activated by load.

The synergy between these growth factors or serum and mechanical load in the stimulation of procollagen synthesis has not been reported in dermal fibroblasts prior to this study. Our laboratory has previously shown the synergistic effects of load and TGFß on rat cardiac fibroblast collagen production (Butt *et al*, 1997). Interestingly, the requirement of growth factors within this system for the synergy response with load is not always typical of fibroblast behaviour. The work of Carver and colleagues (1991) showed increased procollagen $\alpha 1(I)$ gene expression over static controls in cardiac fibroblasts in response to load alone, and this was further enhanced in the presence of IGF-1. Similarly if the same cells are stretched on a fibronectin matrix in

the absence of serum, an increase in procollagen is also seen, which is again enhanced further in the presence of serum (Reynolds *et al*, 1997, abstract).

These data combined would suggest that in these systems it is clear that load consistently increases procollagen synthesis in various cell types. The requirement for growth factors to elicit a synergistic response with stretch appears to be both cell-type and matrix dependent. The different responses seen in different cell types may be explained by variations between adult and fetal primary cells, and transformed cell lines. It is possible that cells exhibiting a very high basal level of procollagen synthesis, such as in transformed lines, are less responsive to growth factors or indeed reach maximal synthesis more rapidly, and therefore mechanical load has no additional effect. Fetal and adult cells may express different levels of growth factor receptors and therefore have altered capacities for procollagen production in response to stimuli. Indeed, it has been shown recently that fetal cells respond in a different manner to TGFß when compared to adult cells, and the reasons for this are under investigation (Cambrey *et al,* 1999).

Extracellular matrix would also appear to be important in governing cellular responses to load. This has been demonstrated in terms of intracellular signalling (MacKenna et al, 1998), proliferation (Wilson 1995) and procollagen synthesis (Reynolds et al, 1998) in mesenchymal cells. The conclusion of these studies is that in each case, plating cells onto fibronectin in the absence of growth factors was sufficient to allow a response to mechanical load in comparison with cells plated on elastin or collagen matrices. Data in this chapter has demonstrated enhanced collagen production in response to load in cells plated on collagen, but only in the presence of serum or growth factors. Whilst other matrices are not investigated in this thesis, by comparing

these findings with those of the above studies, it can be hypothesised that fibronectin may represent a "substitute" for growth factors, thereby promoting a cellular response to load in this system. Fibronectin has been widely reported to be vital in conveying survival and growth signals to cells via the $\alpha5\beta1$ integrin (Dalton *et al*, 1995; Chicurel *et al*, 1997). It is feasible that the responses of cells to load when attached to pure fibronectin only are an extension of these signals, and thereby promote new protein synthesis as a protective measure.

The synergy between mechanical load and growth factors that is demonstrated in this chapter is also a very interesting point. Clearly, growth factors must be present in this system in order for load to stimulate enhanced procollagen synthesis in dermal fibroblasts. There may be a number of reasons for this growth factor requirement. Firstly, fibroblasts may require an initial growth factor stimulus to synthesise a threshold level of procollagen, which then promotes the detection of load. This theory is supported by data indicating that a load response is not detectable until the 48 hour time point, suggesting a delay in the stimulation of collagen synthesis in response to load. The theory is also supported by data in this chapter showing TGFß stimulation of procollagen synthesis after 24 hours, which is enhanced only by load after 48 hours.

Alternatively, growth factors may be required in the first 24 hours of incubation to stimulate a factor or factors required for fibroblast responses to load. For example, the production of an intracellular messenger, cytoskeletal protein or cell-surface receptor that is then involved in mechanotransduction and subsequent enhanced collagen deposition. Similarly, growth factors and load may act together to stimulate a factor required for activation of a load-responsive element in the collagen gene promoter.

For instance, the enhanced response to load in the collagen gene may be stimulated by binding of two separate transcription factors to specific sites on the promoter, the production of which is dependent upon combined signals from growth factors and load. Indeed, it may be a combination of these reasons that allow a fibroblast to respond to mechanical load. In the context of *in vivo* dermal tissue repair, the possible requirement of growth factors to be present to allow fibroblasts to respond to load is logical. Growth factors are only released following injury, and therefore fibroblasts can only deposit enhanced levels of collagen in response to load at this time, when repair is necessary.

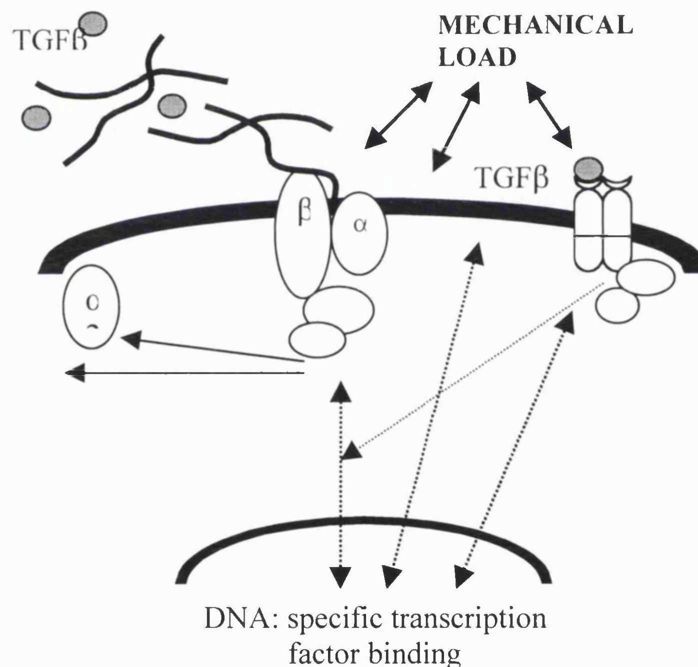


Fig. 3.11:Hypothesised interaction of growth factors and mechanical load in stimulation of procollagen synthesis.

Figure demonstrating how mechanical load and serum growth factors might interact both directly at the transcription level or converging signaling pathways from integrins and growth factor receptors to stimulate new procollagen synthesis in human dermal fiboblasts.

The data presented here would not support a role for this form of soluble autocrine growth factor activity in response to stretch. The study in this chapter using conditioned media from loaded cells highlights the fact that in this system, the increase in procollagen synthesis is not mediated via a soluble factor released into the media. Conditioned media did not stimulate any further increase in fresh fibroblast cultures. This implies that the mechanical load is acting either directly at the gene level or upon intracellular factors, which then translate to an increase in procollagen synthesis. However, it cannot be ruled out that load may be activating other growth factors or their receptors which are important in other cell responses such as changes in cell morphology. Mechanical forces may also stimulate synthesis of a membrane-bound growth factor, which may then act directly back on to the surrounding cells. Alternatively, growth factor production may not be acting via autocrine mechanisms and polypeptides generated in response to stretch may be important in modulating the phenotype of other cells such as keratinocytes or smooth muscle cells which are located *in vivo* adjacent to fibroblasts within the skin.

In conclusion, the data presented here has demonstrated that mechanical load increases procollagen gene expression and protein synthesis in human dermal fibroblasts in the presence of serum, TGFβ or thrombin. This effect is not due to a general increase in cellular protein levels, and is not due to autocrine release of growth factors in response to load. Having established the basic effects of stretch on dermal fibroblast procollagen levels, the further effects of load on processing of procollagen into insoluble collagen, a vital process during in vivo scar formation, will be investigated further in the following chapter.

CHAPTER FOUR

Results:

Regulation of insoluble collagen formation

4.1 INTRODUCTION

The synthesis, export and deposition of procollagen is a highly complex and structured sequence of events. As mentioned previously in chapter 1.3, these events are governed by a number of very important enzymes.

Chapter 1.3 describes how fibril-forming collagens are synthesized as soluble precursors, procollagens, in which the central triple-helical fibril-forming domain, is flanked by amino and carboxyl propeptide domains. Proteolytic removal of the propeptides by specific N- and C-proteinases is essential for the formation of the mature collagen molecule that spontaneously self-assembles into fibrils. In particular, failure to remove the c-propeptides of procollagen is incompatible with fibrillogenesis. Cleavage of the carboxyl propeptides lowers the solubility of procollagen at least 10,000-fold and initiates the self-assembly of fibrils (Prockop and Hulmes, 1994; Kadler and Watson, 1995). PCP has been isolated as two distinct molecular weights (80 and 110 kDa). It is now known that these represent distinct products of the same gene, referred to as BMP-1 and mTld, or pCP1 and pCP2 respectively (Li *et al*, 1996; Takahara *et al*, 1994). Early studies also lead to the discovery of the procollagen C-proteinase enhancer protein (PCPE), a glycoprotein that binds to the c-propeptide of procollagen type I and enhances procollagen processing by PCP dramatically (Kessler *et al*, 1986; Kessler and Adar, 1989). Thus, procollagen C-proteinase and its enhancer protein play critical roles in collagen deposition.

PCP and PCPE are recognized as being key in the formation of a stable collagen matrix in both development and genesis of adult tissues. PCP is found mainly in bone,

cartilage and skin, where fibrillar collagen is in abundance (Kadler and Watson, 1995). The importance of PCP in regulation of collagen deposition is highlighted by the connective tissue defects seen in mouse embryos lacking the *Bmp-1* gene (Suzuki *et al*, 1996). Homozygous mutant embryos display partially processed procollagen of abnormal morphology within the extracellular matrix (ECM) of structures including the amnion, leading to herniation of the gut and consequently they do not survive beyond birth. Homozygous mutant fibroblasts in culture also revealed abnormal processing and deposition of collagen.

Little is known about the regulation of PCP expression. Recent studies suggest however that PCP expression may be coordinately regulated with that of collagen type I. The addition of TGFß to cultures of MG63 fibrogenic cells or keratinocytes was found to increase the levels of BMP-1 and mTld mRNAs up to seven-fold, in parallel to the increase in collagen $\alpha 1(I)$ mRNA. This stimulatory effect was more pronounced in the presence of ascorbic acid and the increase in mRNA levels was mirrored by elevated levels of BMP-1 and mTld proteins in the media as well as increased procollagen processing (Lee *et al*, 1997). This regulation of procollagen and PCP by TGFβ may represent more than a simple direct correlation between procollagen and PCP expression. TGFß stimulates the biosynthesis of numerous structural matrix proteins as well as upregulating lysyl oxidase expression (Massague, 1990; Feres-Filho *et al*, 1995). BMP-1 was initially isolated in a complex with other BMPs that are members of the TGFß superfamily and presumed to have the capacity to induce bone formation. It was hypothesised therefore, that the bone-inducing activity of BMP-1 may result from its ability to proteolytically activate latent forms of

TGFß-like BMPs (Wozney *et al*, 1988). This theory has yet to be proven, but forms a very interesting hypothesis for the mode of action of BMP-1/PCP.

There is less known about the regulation of the PCP enhancer protein (PCPE). PCPE is abundant in connective tissues rich in collagen I but undetectable in brain and liver (Takahara *et al*, 1994; Ogata *et al*, 1997). However, PCPE is expressed in fibrotic livers of CCl_4-treated rats. The expression of PCPE mRNA in cultured liver stellate cells derived from these rats is upregulated by TGFß and down-regulated by tumor necrosis factor α (TNF-α), in parallel to changes in the levels of α1(I) procollagen mRNA (Ogata *et al*, 1997). In cultured fibrogenic cell lines and keratinocytes, the addition of TGFß had no effect on PCPE expression (Lee *et al*, 1997). Correlation of PCPE expression with collagen *in vivo*, along with the coordinated upregulation of both proteins in response to growth factors in vitro highlights the importance of PCPE in regulation of collagen deposition.

PCPE expression also seems to affect cell growth properties. Rat fibroblasts expressing decreased amounts of PCPE as a result of retroviral vector integration into the PCPE gene display a phenotype similar to that of a malignantly transformed cell. They lose contact inhibition, show altered morphology and can grow in an anchorage-independent manner (Masuda *et al*, 1998). The normal phenotype is restored upon transfection of the mutant cells with a PCPE expression plasmid. The mechanism by which PCPE affects cellular growth is unclear. Decreased type I collagen synthesis is known to be associated with malignant cellular transformation (Arbogast *et al*, 1977) and restoration of collagen synthesis can suppress the malignant phenotype (Travers *et al*, 1996).

Therefore, although there is comparatively little known about PCP and PCPE, it has been well established that they are critical in the formation of insoluble collagen both in pathological scenarios, and in normal tissue repair and turnover.

Aims

The aims of this chapter are to assess the effects of mechanical load in the presence of serum and TGFß on insoluble collagen deposition by primary human dermal fibroblasts. Levels of PCP and PCPE mRNA and protein are also investigated in the same cultures, in order to assess if these enzymes are regulated by mechanical load in parallel with procollagen expression. As there are no chemical inhibitors of PCP currently available the importance of PCP activity in the load-induced stimulation of collagen deposition is tested using a biological inhibitor of PCP, L-arginine.

4.2 RESULTS

4.2.1 Mechanical load in the presence of 10% FCS and TGFß enhances insoluble collagen deposition.

Fig. 4.1 shows the effects of mechanical load with and without growth factors on insoluble collagen deposition over 48 hours. Under serum-free conditions, insoluble collagen was not detectable until 24 hours of incubation. Insoluble collagen was just detectable at 8 hours in the presence of growth factors. Mechanical load alone had no effect on insoluble collagen deposition.

Serum and TGFß increased the level of insoluble collagen compared to serum-free controls by 220±7% ($p<0.001$) and 158±6% ($p<0.001$) after 36 hours respectively and by 340±9% ($p<0.001$) and 300±7% ($p<0.001$) after 48 hours. Mechanical load enhanced the levels of insoluble collagen deposited under these conditions by 36 hours and further increased deposition at 48 hours. By 48 hours load enhanced deposition by 124 ± 8% ($p<0.01$) in serum-treated cultures and in TGFß-treated cultures by 115±9% ($p<0.01$) compared with growth factor controls. Thrombin did not significantly enhance the deposition of insoluble collagen in the presence or absence of load. There was no significant difference between collagen deposition in control vs. loaded cultures at any of the time points investigated.

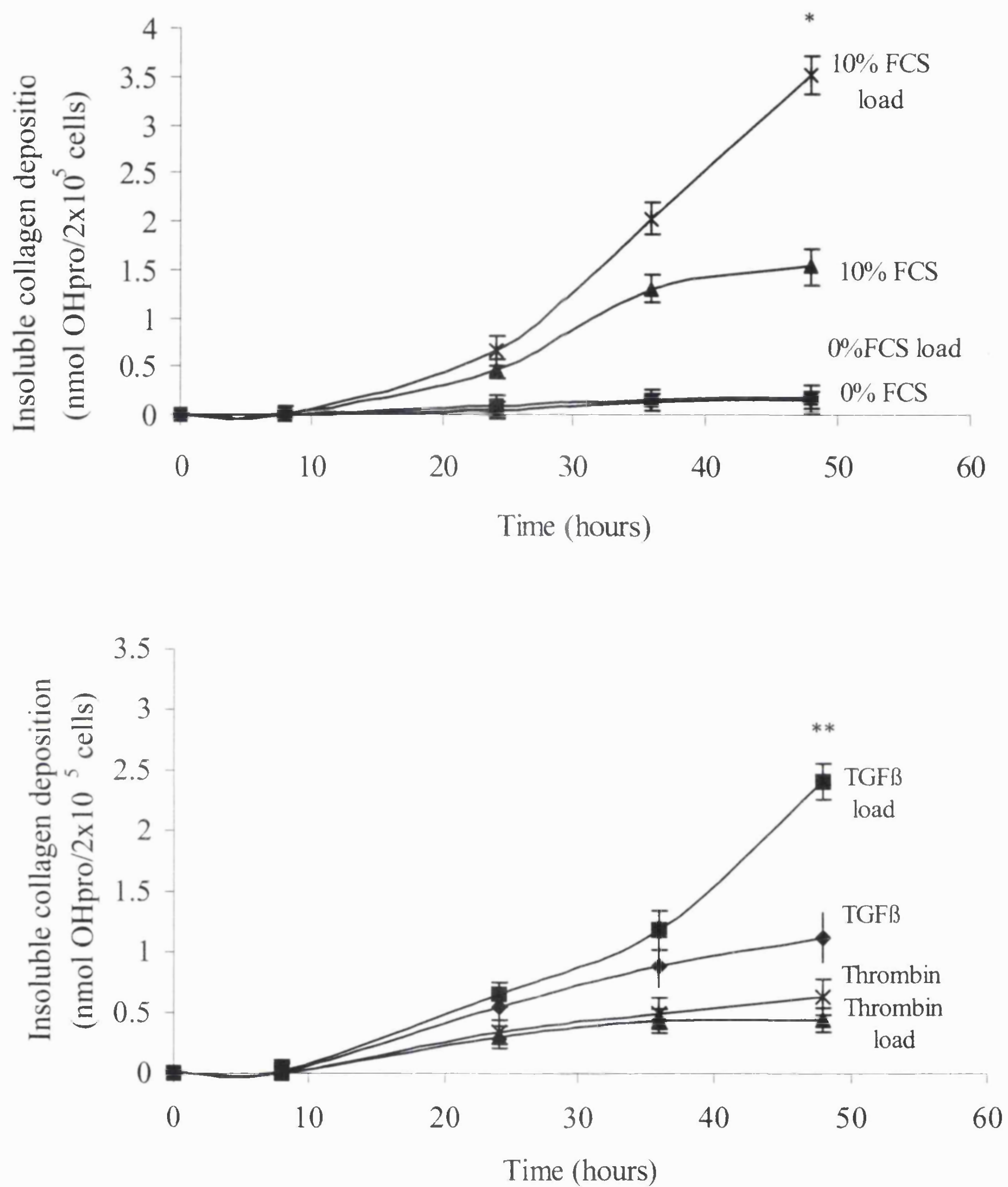


Fig. 4.1: Mechanical load in the presence of 10% FCS and TGFß enhances insoluble collagen deposition.

The deposition of insoluble collagen in response to growth factors and mechanical load was assessed using HPLC to measure levels of hydroxyproline in insoluble protein fractions over 48 hours. Insoluble collagen is represented as nmol OHpro/2x10^5 cells. All values are corrected for changes in cell number. * denotes $p<0.001$, ** denotes $p<0.01$level of significance compared to growth factor control .

4.2.2 Mechanical load in the presence of 10% FCS or TGFβ enhances procollagen C-proteinase (PCP) mRNA expression

Figure 4.2 shows PCP mRNA levels in response to serum and TGFß with and without the application of mechanical load. Under serum-free conditions mechanical load alone had no effect on PCP expression. However, both serum and TGFß alone enhanced PCP mRNA levels 100±30% ($p<0.01$) and 80±24% ($p<0.01$) respectively. In the presence of mechanical load, 10% FCS mediated a 104±30% increase in PCP expression after 24 hours over FCS control. TGFß had a similar effect, with load stimulating PCP mRNA levels by 130±20% under these conditions above TGFβ alone conditions. PCP mRNA was only enhanced in response to load after 24 hours of culture, after which time expression returned to respective control levels.

4.2.3 Serum and TGFβ in the presence of mechanical load increases PCP protein levels.

PCP protein expression is shown in Fig 4.3 under identical conditions to those described above. 10% FCS and TGFß enhanced PCP protein levels by 120±9% and 95±13% respectively compared to serum-free controls. Mechanical load further enhanced PCP protein expression by 140±35% and 100±32% respectively in serum and TGFß treated cultures above growth factor controls. At the 48 hour time point, there were no differences in PCP protein expression between loaded and respective growth factor control samples.

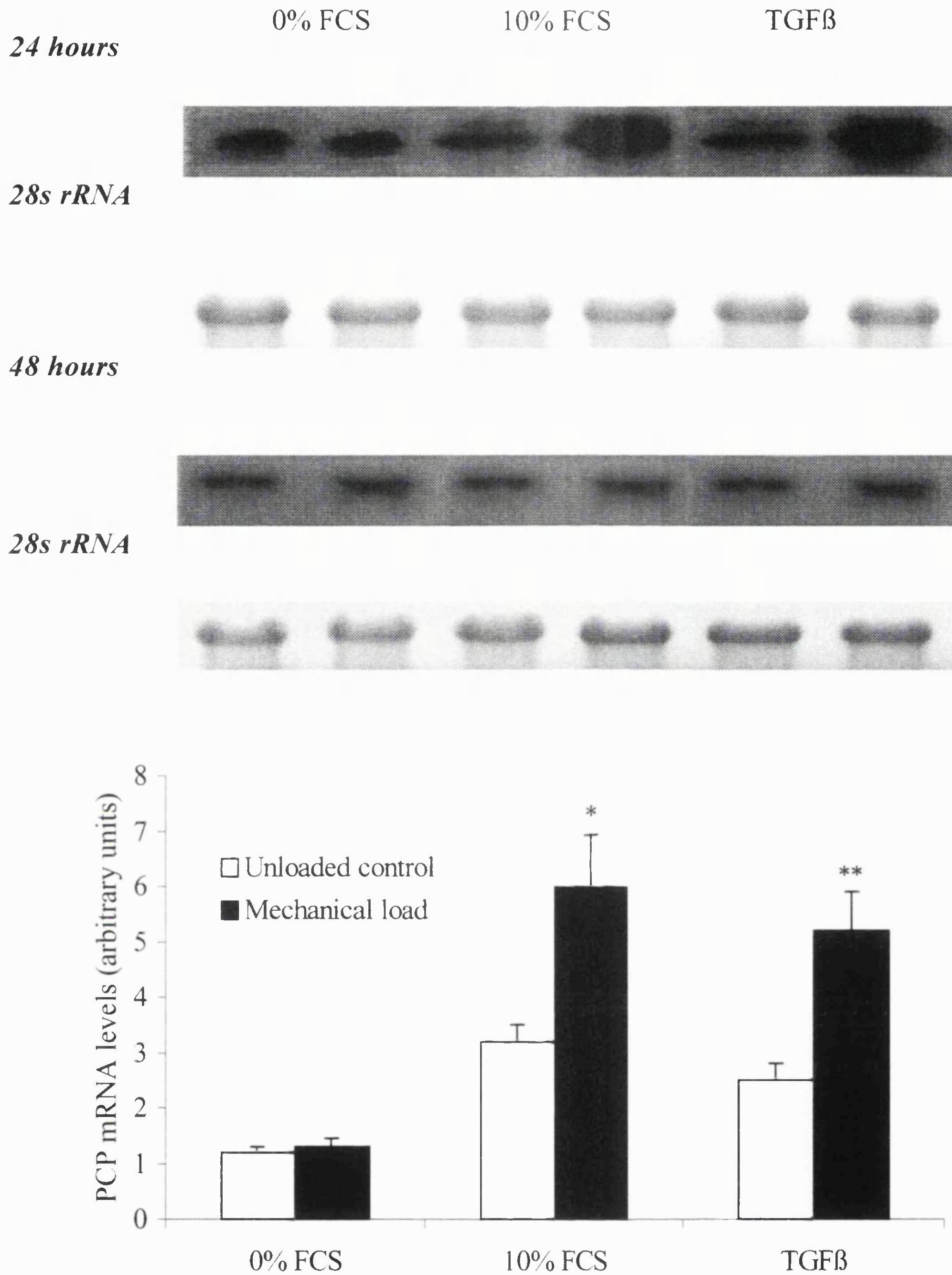


Fig. 4.2: Growth factors in the presence of mechanical load stimulates procollagen c-proteinase (PCP) mRNA expression.

The figure shows northern blots of PCP mRNA in response to mechanical load and serum or TGFß over a time course of 48 hours, and corresponding 28s rRNA loading controls. Quantitation of PCP mRNA by densitometric scanning is shown in the bottom panel at the optimal time point of stimulation, 24 hours. * denotes $p<0.001$ and ** $p<0.01$ compared to respective controls. n=5.

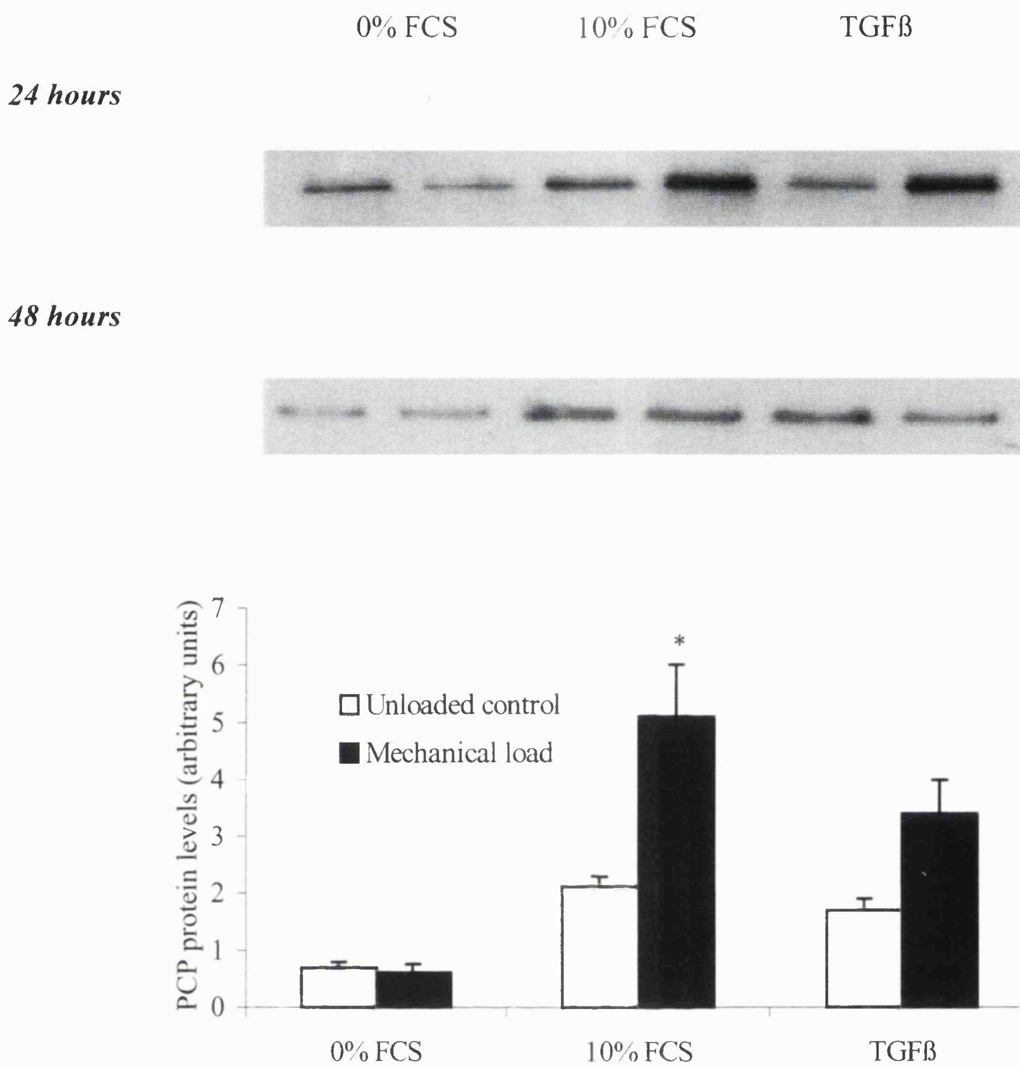


Fig. 4.3: Serum and TGFβ in the presence of mechanical load increases PCP protein levels.

Western blot (top panels) showing PCP protein levels following 24 and 48 hours incubation with serum or TGFß with and without mechanical load. Densitometric scanning quantitation (bottom panel) of western blots at optimal time of 24 hours only. *denotes $p<0.001$ level of significance of loaded samples compared with respective unloaded control. n=5.

4.2.4 Serum and TGFβ stimulates increases expression of procollagen c-proteinase enhancer protein (PCPE) mRNA, load has no additional effect.

The data in figure 4.4 shows the expression of PCPE mRNA in response to serum and TGFß with and without the application of mechanical load. PCPE mRNA expression was upregulated in response to both 10% FCS and TGFß by 205±42% and 190±38% respectively compared to serum-free controls after 24 hours. Load had no additional effect on PCPE mRNA levels at either 24 or 48 hour time points in any of the cultures.

4.2.5 Serum and TGFβ stimulates increased PCPE protein expression, load has no additional effect.

PCPE protein levels in identical cultures to those described above are shown in Fig 4.5. Protein levels were enhanced by 350±65% and 305±70% in serum and TGFß treated cultures respectively after 24 hours. PCPE protein levels were still elevated in these cultures after 48 hours. Load had no additional effect on PCPE protein levels in any of the cultures at any time point.

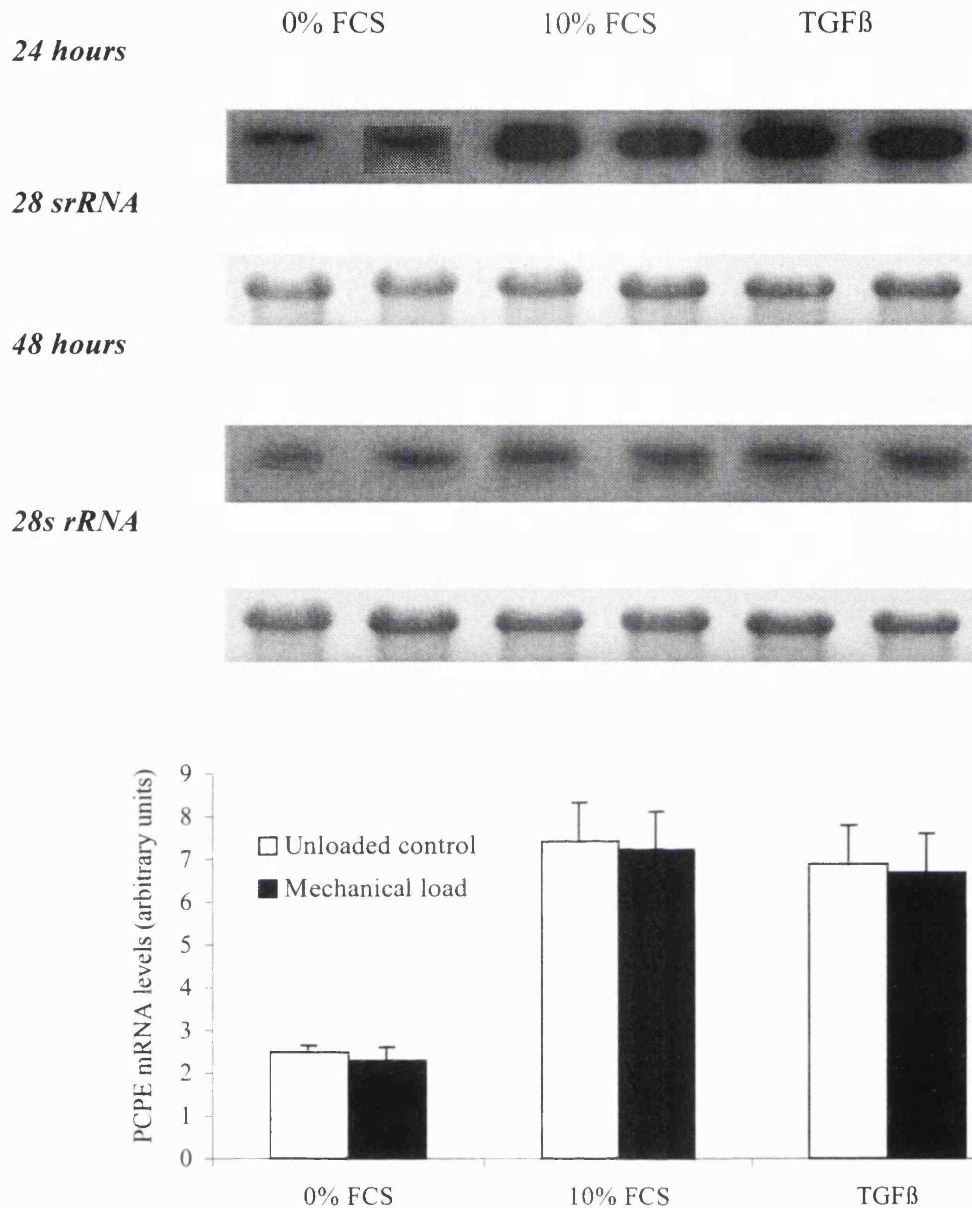


Fig. 4.4: Serum and TGFβ stimulates increased expression of procollagen c-proteinase enhancer protein (PCPE) mRNA, load has no additional effect.

The figure shows northern blots of PCPE mRNA in response to mechanical load and serum or TGFß over a time course of 48 hours, and corresponding 28s rRNA loading controls. Quantitation of PCPE mRNA by densitometric scanning is shown in the bottom panel at the optimal time point of stimulation at 24 hours. * and ** denotes $p<0.001$ and $p<0.01$levels of significance compared to respective control. n=5.

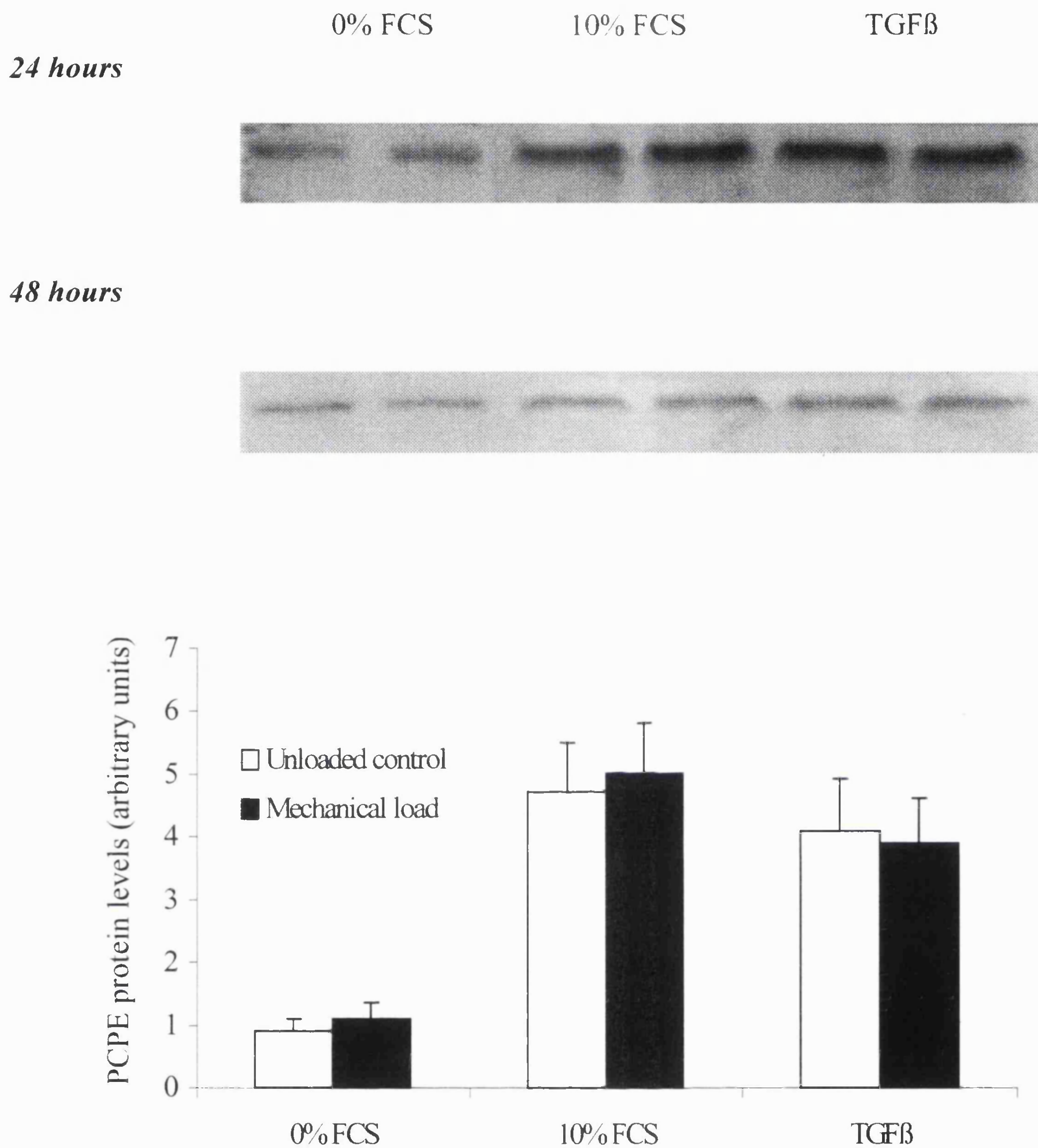


Fig. 4.5: Serum and TGFβ stimulate increased PCPE protein levels, load has no additional effect.

Western blot (top panel) showing PCPE protein levels (bottom panel) following 24 and 48 hours incubation with serum and TGFß with and without mechanical load. Densitometric scanning quantitation (bottom panel) of western blots at optimal time of 24 hours only. *denotes $p<0.001$ level of significance compared to respective unloaded control. n=5.

4.2.6 Insoluble collagen deposition in response to load is inhibited by L-Arginine but unaffected by a pan-MMP inhibitor.

Figure 4.6 shows the effect of L-Arginine and the broad spectrum MMP inhibitor BB3103 on procollagen processing following 24 and 48 hours of mechanical loading in the presence of 10% serum. L-Arginine has been shown previously to inhibit PPC activity in vitro. BB3103 inhibits MMP activity, but does not affect PCP activity, thereby distinguishing procollagen processing from degradation. No significant increase in insoluble collagen deposition was seen after 24 hours of load, and no significant differences in procollagen processing were observed in the presence of either compound. After 48 hours of mechanical load in the presence of 10% serum, mechanical load stimulated a 145±12% increase in insoluble collagen deposition. Collagen deposition in both the loaded and unloaded was unaffected by the presence of the pan-MMP inhibitor BB3103 at 10 μM. However, L-Arginine inhibited the load-induced insoluble collagen formation without affecting serum control levels, reducing the collagen in loaded samples to 10% serum control levels.

Table 4.1 shows procollagen synthesis and processing in the presence and absence of L-Arginine and BB3103 following 48 hours loading in the presence of 10% serum. L-Arginine prevented the increase in insoluble collagen deposition due to 48 hours of mechanical load without affecting total procollagen synthesis or degradation. Consistent with this, higher amounts of soluble procollagen were found in these cultures, indicating that L-Arginine has indeed blocked the procollagen processing. No effect was seen on levels of insoluble collagen when cultures were incubated in the presence of a broad spectrum MMP inhibitor BB3103.

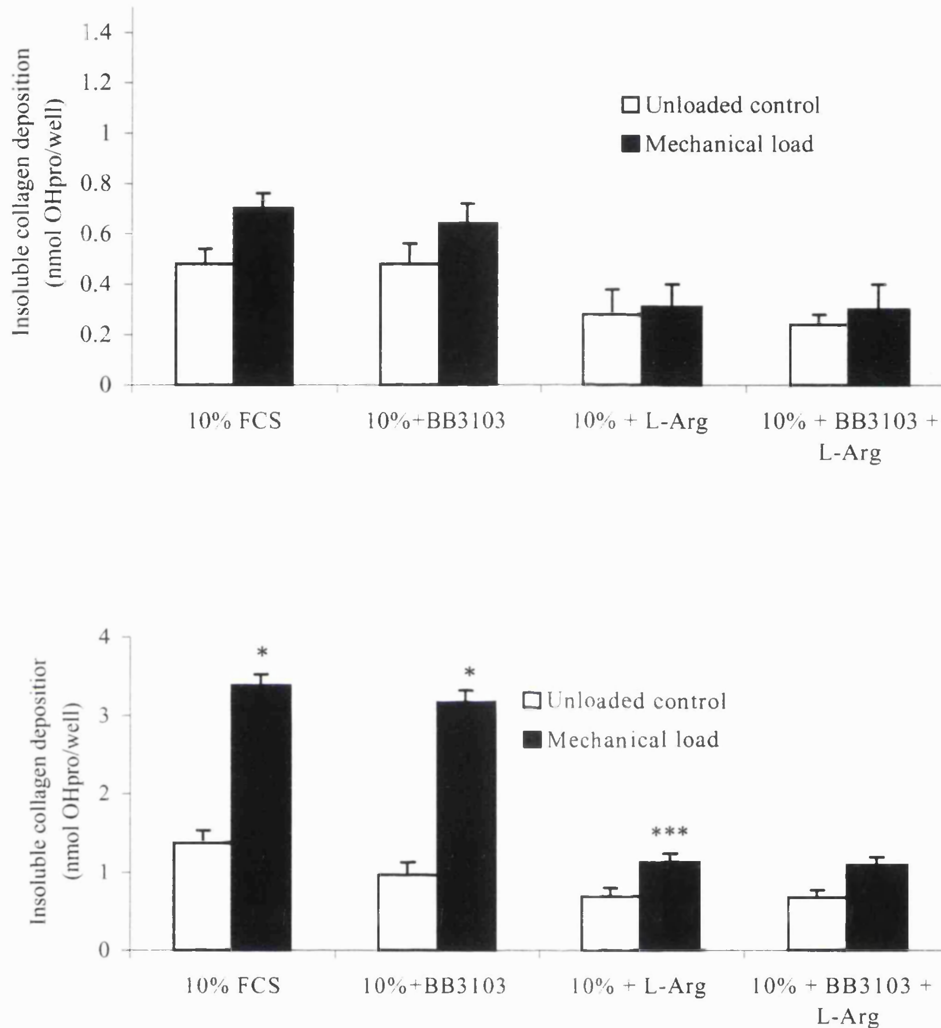


Fig. 4.6: Insoluble collagen deposition in response to load is inhibited by L-Arginine but unaffected by a pan-MMP inhibitor.

L-Arginine was used as an inhibitor of the proteolytic activity responsible for the cleavage of the c-terminal propeptide. BB3103, a broad range MMP inhibitor was used to control for non-specific matrix degradation, which may generate insoluble collagen. Each condition was repeated at least 3 times at 24 (top panel) and 48 hours (bottom panel). *denotes $p<0.001$ and *** denotes $p<0.05$ between loaded vs. unloaded growth factor control.

	10% FCS		+L-Arginine (50nm)		+BB3103		+BB3103 and L-Arginine	
	Control	Load	Control	Load	Control	Load	Control	Load
Total Procollagen Synthesis (nmol OHpro/well)	**5.96 ±0.16**	**11.35 ±0.14***	**6.69 ±0.14**	**11.73 ±0.2***	**5.68 ±0.16**	**12.00 ±0.13***	**6.78 ±0.12**	**14.04 ±0.16***
Total Degradation (nmol OHpro/well)	**1.2 ±0.1**	**1.6 ±0.44**	**1.4 ±0.09**	**1.1 ±0.12**	**1.2 ±0.32**	**1.1 ±0.11**	**1.0 ±0.2**	**2.75 ±0.2**
Soluble Procollagen (nmol OHpro/well)	**3.2 ±0.12**	**6.9 ±0.12***	**4.6 ±0.16**	**9.5 ±0.1***	**3.5 ±0.14**	**7.7 ±0.07***	**5.1 ±0.1**	**10.2 ±0.15***
Insoluble Collagen (nmol OHpro/well)	**1.36 ±0.07**	**3.45 ±0.13***	**0.69 ±0.16§§**	**1.13±0.14**§**	**0.98 ±0.14**	**3.2 ±0.12***	**0.68 ±0.22§§**	**1.09 ±0.1**§**
Insoluble Collagen (as% of total synthesis)	**22 ±1%**	**31 ±1%****	**11 ±2%§§**	**9 ±1%§**	**18±3%**	**27 ±2%****	**10 ±1%§**	**8±1%§**

Table 4.1: Insoluble collagen deposition in response to mechanical load is inhibited by L-Arginine

Insoluble collagen deposition was measured in the presence of L-Arginine and BB3103 (pan-specific MMP inhibitor). The effect of the two compounds, both separately and combined, on procollagen processing were assessed. All experiments were conducted in the presence of 10% FCS after 48 hours. Total procollagen synthesis was separated into soluble procollagen and insoluble collagen deposition, and total degradation was taken to be free OHpro in the soluble fraction. Results are expressed as nmol OHpro/well unless otherwise stated. Each condition was repeated 3 times with similar results. N=6. * denotes $p<0.001$ and ** denotes $p<0.05$ level of significance between loaded and unloaded growth factor control. § denotes $p<0.001$ and §§ denotes $p<0.05$ level of significance compared to serum alone control equivalent.

4.3 DISCUSSION

In this chapter, the studies have focussed on the elucidation of the effects of stretch on procollagen processing, and the enzymes involved in this process. Previous studies on the effects of mechanical load on procollagen synthesis have concentrated on transcriptional regulation or procollagen protein production (Carver *et al*, 1991; Butt and Bishop, 1997). The effects of load on processing of procollagen, procollagen c-proteinase and respective enhancer protein have not been investigated prior to this thesis.

In this chapter it was shown for the first time that exogenous mechanical load can enhance the processing of procollagen to insoluble collagen, observed as an increase in the actual amount of insoluble collagen produced, and as a greater proportion deposited of the total procollagen synthesized. It was also demonstrated that TGF-β and serum are potent stimulators of both PCP and PCPE - the enzymes involved in procollagen processing, and that these enzymes are stimulated at an earlier time point than the increase in procollagen synthesis. PCP mRNA and protein, but not PCPE are further increased by mechanical load.

PCP is a neutral metalloproteinase, and it is possible that the response to load also involves upregulation of the matrix metalloproteinases. It was therefore necessary to rule out any non-specific cleavage of procollagen peptides by the matrix metalloproteinases in this system. Firstly, L-Arginine was used as an inhibitor of procollagen c-terminal pro-peptide cleavage (Leung *et al*, 1979). This effectively blocks the processing of procollagen by up to 80% (Kessler *et al*, 1996). Fig. 4.6 in

this chapter demonstrates that L-Arginine was able to block the load induced stimulation of insoluble collagen deposition. L-Arginine is involved in the production of nitric oxide (NO), and some studies have previously reported decreased procollagen synthesis in the presence of NO (Schaffer *et al*, 1997). However, in this study, the L-Arginine used to inhibit procollagen processing does not affect procollagen synthesis in the cultures in the studies in this thesis (see Table 4.1). The broad spectrum MMP inhibitor BB3103 was also used, as it selectively inhibits MMP's at this concentration, but will not inhibit PCP activity. The MMP inhibitor had no effect on insoluble collagen deposition in response to load. The collagen deposition would therefore seem to be specifically dependent on increased PCP levels. Evidence that MMP activity was not increased by load in this study came from analysis of free OHpro levels - an index of collagen breakdown products. The few studies conducted in this area have shown that load may influence MMP activity, but the effect appears cell-type specific, and depends on loading conditions (Lambert *et al*, 1992; Lambert *et al*, 1998; Yasuda *et al*, 1996; Yang *et al*, 1998). In the present study, the MMP inhibitor did not influence the level of procollagen degradation suggesting that procollagen degradation occurs predominantly intracellularly (via the action of lysosomal proteinases) in these cell culture conditions (Bienkowsky and Engels, 1981; McAnulty *et al*, 1991).

The results presented here indicate a growth factor (e.g.: serum or TGF-ß) must be present to detect any expression of PCP and PCPE. PCPE levels are then not increased beyond this level by mechanical load, whereas PCP levels are elevated further by load. This would suggest a different mechanism of regulation for each of the genes. There is very little currently known about the PCP or PCPE promoters in

terms of important consensus regulatory sequences. However, it is known that TGF-ß can increase expression of PCP both *in vitro* and *in vivo* (Lee *et al*, 1997; Greiling et al, 1999). The findings of the study by Lee and colleagues would strongly imply similar regulatory mechanisms in the *BMP-1* gene to those found in the procollagen α_1(I) gene. In this study, both TGFß and ascorbate stimulated expression of both PCP and the related splice variant mTld after 12 hours, and this event was dependent on new protein synthesis. Procollagen processing was enhanced following PCP upregulation after 24 hours. It would appear therefore that based on evidence in this thesis and other studies, both PCPE and PCP are regulated by growth factors, but respond differently to mechanical load.

There is a very feasible hypothesis for this differential regulation. PCP is expressed at very low levels, even in cultures stimulated with mechanical load and growth factors. PCPE however, is abundant all in cultures treated with growth factors, and where present acts to enhance any PCP activity. It may be, therefore, that PCPE expression is less regulated than PCP, as it does not represent a rate-limiting factor in the formation of insoluble collagen. PCPE has no activity if PCP is not present in the immediate environment (Kessler *et al*, 1989), and therefore is inactive until PCP levels are increased by a limiting factor, such a stretch. The fact that PCPE expression is only upregulated by growth factors suggests that PCPE is not required at high levels for normal collagen turnover *in vivo,* where high levels of cytokines are not usually available. However, following dermal injury, growth factor levels are high and the requirement for deposition of new collagen is more urgent, so PCPE levels rise, possibly to a maximal level, to accelerate this process. The more intricate regulation

of collagen deposition is achieved by strict temporal regulation of PCP by multiple factors such as load, TGFß and serum.

The time taken to observe the responses seen in mechanically loaded cultures is also an interesting point. Increases in collagen deposition in response to load and growth factors are not seen until minimum 36 hours incubation, with significant differences seen in both soluble and insoluble collagen productions after 48 hours. The reason for the delay is not clear but may suggest the necessity for synthesis of an intermediary in the response. The inability of load-conditioned media to stimulate new collagen synthesis as shown in chapter 3 have indicated that this is unlikely to be a soluble factor secreted into the media. Interestingly, PCP levels are increased in response to load and serum after only 18 hours, with maximal PCP expression seen at 24 hours, decreasing by 48 hours. These differences would suggest that the regulation is at least partially independent of the procollagen gene expression response. This is an interesting observation as it implies separate or more rapid regulation by mechanical load of the collagen processing enzymes compared to the procollagen genes.

There are a number of potential reasons for the differential regulation of PCP and procollagen synthesis. PCP is upregulated much faster than procollagen in response to load and growth factors. This may be because as procollagen levels continue to rise, PCP transcription is rapidly enhanced in order to generate enough processing enzyme at the cell surface to cleave and deposit the newly secreted procollagen molecules. Indeed, procollagen synthesis itself may be involved in the regulation of PCP levels (Lee *et al,* 1998). It is possible that once a threshold level of procollagen synthesis is achieved, a feedback mechanism stimulates rapid synthesis of PCP. Once PCP has

been generated to process the procollagen, transcription returns to basal levels, as is seen in this chapter in response to mechanical load. It is also possible that the downregulation of PCP in the presence of load at the 48 hour time point is regulated by an external factor, such as receptor occupation. This may include specific growth factor receptors or indeed collagen-binding integrins which have been shown to be important in regulating procollagen synthesis (Riikonen *et al*, 1995). In this case, whilst the same factors are responsible for stimulating PCP and procollagen synthesis, discrete factors are involved in the downregulation process, thereby allowing temporal regulation to occur.

Therefore, this chapter presents evidence to suggest that mechanical load is important *in vitro* in the regulation of procollagen synthesis and processing. There is currently no data available from studies conducted *in vivo* on the importance of mechanical load in wound healing and scar formation. Likewise, no information is available on the levels or activity of PCP enzyme in scar formation. Therefore, it can only be hypothesized as to the relevance of data gained *in vitro* to an *in vivo* scenario. As PCP is the only known enzyme capable of specific c-propeptide cleavage of procollagen, it may be assumed that this is the enzyme involved *in vivo*. Clinical studies support the argument that mechanical stretch can greatly influence the size of a scar, although the biochemical basis for this in terms of matrix deposition is unknown (Elliot and Mahaffey, 1991). Therefore it could be hypothesized that load is acting in synergy with growth factors present at the site of wound repair, and is directly increasing procollagen synthesis and PCP activity. As this tension is continually present throughout the process of healing from surrounding movement of dermis, the amount of collagen deposited is increased, and a scar is formed. The data in this chapter also

indicates that the load-induced upregulation of procollagen processing events is transient, therefore ensuring the deposition of collagen is not uncontrolled and ongoing, as is the case in hypertrophic or keloid scarring (for review see Clark, 1993; McGrouther, 1994). However, the mechanisms responsible for the deposition of collagen in response to load are unknown. The potential role of the cell-matrix receptors, integrins in these responses is investigated in the following chapters.

CHAPTER FIVE

Results:

Regulation of integrin subunit expression

5.1 INTRODUCTION

It has been widely hypothesised in recent literature that mechanical load acts to stimulate intracellular effectors via the mediation of the cell-surface receptors integrins. Whilst this theory is not definitively proven it is supported by a large amount of circumstantial evidence. The effects of growth factors and extracellular matrix have been previously studied in the context of integrin signalling, and indeed control of integrin transcription, assembly and recruitment to the cell membrane. However, the precise effects of mechanical forces on integrin function and assembly are currently poorly understood. Further understanding of these processes is vital to assist in the elucidation of cell-surface mechanoreceptors.

Not unexpectedly, different integrin subunits are regulated by different factors. The ß1 integrin subunit, which is a generic subunit binding to one of ten alpha subunits, has been shown to be regulated principally from a large intracellular precursor pool, which takes around 10 hours to mature to the final glycoprotein form. It would appear that during normal metabolism of this integrin there is massive pre-golgi degradation of the ß1 precursor which is sensitive to protease inhibitors and ATP depletion (Vekeman *et al*, 1993). Very few studies have investigated cell surface cycling of integrins. However, it is hypothesised that ß1 receptors disappear from the cell surface by shedding of the entire membrane fragments (exosomes), or by proteolytic degradation at the cell surface (Vekeman *et al*, 1993). The detailed mechanisms are still under investigation.

A number of reports have documented the effects of growth factors on different

integrin α and β subunit regulation. TGFß1 displays concomitant regulation of the $\alpha1$, $\alpha2$, $\alpha3$ and $\alpha5$ subunits that all share the common ß1 subunit. In lung fibroblasts, TGFß1 elevates all the aforementioned α subunits at both the protein and mRNA level, their assembly into the corresponding $\alpha\beta1$ complexes and their exposure on the cell surface (Heino *et al*, 1989). The rate of synthesis of total α subunits relative to $\beta1$ subunit was higher in TGFß treated cells than control cells. The rate of maturation of the $\beta1$ subunit from the precursor was also decreased from 10 hours to 3 hours. The regulation of subunits by TGFß was shown to be via parallel but independent mechanisms (Heino *et al*, 1989). These studies indicate that TGFß, as well as being a highly potent profibrotic agent, is a very important factor in the regulation of integrin receptors.

Another study has demonstrated that PDGF can also regulate levels of $\alpha2$, $\alpha3$ and $\alpha5$ integrin subunits in fibroblasts cultured in different three-dimensional matrices. Fibronectin-rich cultures and fibrin gels supported PDGF-BB induction of $\alpha5$ mRNA. In contrast, both stressed and relaxed collagen gels attenuated these responses while promoting maximal $\alpha2$ mRNA expression (Xu and Clarke, 1996). Post-transcriptional regulation was an important mechanism in this differential response. PDGF-BB and collagen gels together promoted $\alpha2$ but not $\alpha3$ and $\alpha5$ mRNA stability. Conversely, fibroblasts in fibronectin-rich cultures showed enhanced $\alpha3$ and $\alpha5$ mRNA stability, but not $\alpha2$ (Xu and Clark, 1996). This data highlights the interaction between growth factors and ECM, which has been shown to be critical in the control of integrin types and levels.

It is well appreciated that alterations in ECM-integrin interactions cause changes in

cell shape and behaviour. Many investigations have attempted to segregate the effects of the ECM on cell morphology from integrin- and growth factor-derived biochemical signalling and concomitant changes in gene expression. However, recent studies have demonstrated that ECM-dependent changes in cell shape and three-dimensional tissue architecture can determine cell function by modulating both integrin expression and signalling pathways. For example, a role for cell shape impacting upon integrin-dependent signalling pathways has also been demonstrated in angiogenic endothelial cells, which degrade their existing basement membrane and proliferate in response to basic FGF. The proliferation and subsequent survival of the endothelial cell depends upon adhesion to a provisional ECM via the αvβ3 integrin, as blockade of αvβ3 integrins results in a failure to proliferate due to unscheduled apoptosis (Brooks, Clark and Cheresh, 1994). However, occupation and ligation of αvβ3 integrins using anti-integrin antibodies fails to support endothelial cell proliferation and survival if the cells are prevented from acquiring a spread morphology (Re *et al*, 1994).

These studies all indicate that cell shape, which in many systems is comparable to mechanical loading, is very important in both governing integrin responses to ECM and indeed the effects of cell shape on cell proliferation and survival. In order to understand further how such integrin-ECM dependent cell shape models operate to control cell behaviour, it will be necessary to identify additional proteins that are able to co-ordinate both intracellular signalling pathways, and cell shape. To this end, the recent discovery that the Rho family of small GTPases are able to relay integrin-derived signals, as well as organise the actin cytoskeleton suggests that these proteins are well poised to integrate cell shape and function (Hill, Wynne and Treisman, 1995). The downstream consequences of changes in integrin expression in terms of cytoskeletal structure and signalling and are still very poorly understood.

There have been very few reports assessing the direct effects of changes in cell shape and application of mechanical load on integrin expression and reorganisation. The principle findings have demonstrated that 2-dimensional load can both upregulate and re-organise different integrins on endothelial cells. The ß3 integrin subunit was found to be increased both at mRNA and protein levels in human umbilical endothelial cells (HUVECs) exposed to cyclic stretch on fibronectin-coated plates. The number of integrin ß3 adhesions increased in stretched HUVECs compared to non-stretched cells (Suzuki *et al*, 1997).

Another report showed that HUVECs under cyclical strain displayed ß1 integrin reorganisation in a linear pattern parallel with the long axis of the elongated cells creating a fusion of focal adhesion plaques in cells plated on either fibronectin or collagen coated plates. Cyclic strain also lead to a re-organisation of α5 and α2 integrins in a linear pattern in HUVECs seeded on fibronectin or collagen respectively. The actual expression of integrins α5, α2 and β1 did not change at the mRNA or protein level in any of the cultures exposed to strain as compared to their respective controls (Yano, Geibel and Sumpio, 1997). Another study assessed effects of load in osteosarcoma cells with respect to ß1 integrin sub-unit only. In this system, ß1 integrin mRNA and protein levels were increased in response to cyclic mechanical deformation, and the concentration of ß1 localised in cell-surface focal adhesion clusters was increased in stretched cultures (Carvalho *et al*, 1995). These studies demonstrate the importance of cell surface stresses in regulating integrin expression.

All these studies indicate that mechanical forces are obviously important in governing

the cellular integrin make-up, and that this differs to some extent depending upon the type of matrix the cells are adhered to. However, no studies have addressed the functional role, if any, of these changes in integrin expression. The changes in integrin composition and effects on intracellular signalling pathways, and subsequent biochemical events are still under investigation.

Aims

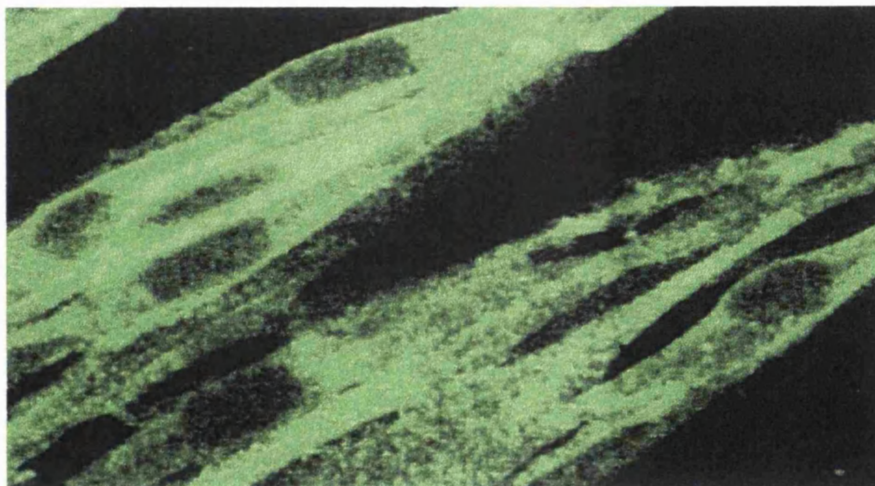
The aims of this chapter were to assess the changes in integrin expression in human dermal fibroblasts exposed to cyclic mechanical strain in the presence of serum and TGFß. The expression of integrin subunits α1, α2, α5 and the generic β1 subunit are assessed in this system, as these integrins have been shown previously to be important in mesenchymal cell responses to load. However, the effects of mechanical forces on integrin expression in dermal fibroblasts have not been investigated prior to this thesis. Total expression of these integrin subunits is measured to ascertain any changes in basal or growth factor regulated protein levels in response to stretch. Cell–surface expression of these subunits is also investigated using biotinylation and immunoprecipitation, to ascertain if cyclical load or growth factors stimulate recruitment of these integrins to the membrane.

5.2 RESULTS

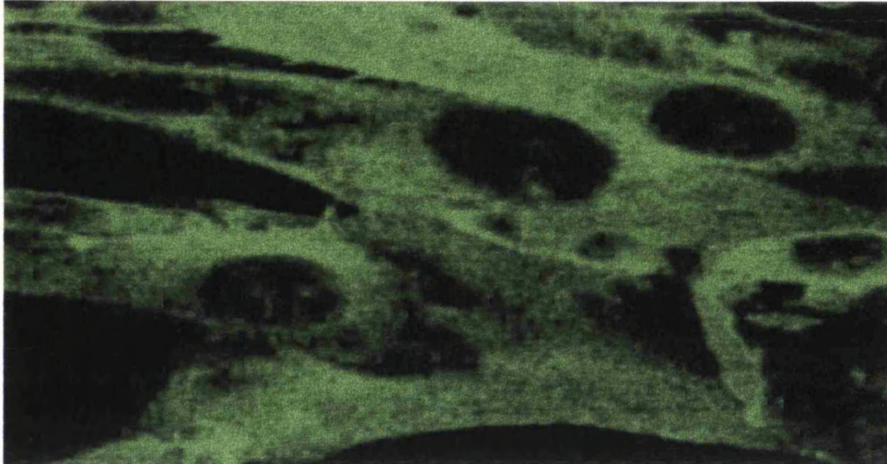
5.2.1 Visualisation of intracellular and membrane-bound integrin subunits in primary human dermal fibroblasts

The confocal micrographs below indicated that α1, α2 and α5 integrin subunits were expressed in the isolated primary human dermal fibroblasts used throughout this study. Whilst the technique does not allow absolute quantitation of levels of these subunits, micrographs taken at the same laser intensity levels show much higher staining for α1 and α2 subunits compared with the α5 subunit. Controls stained with secondary conjugate only displayed no background fluorescence (data not shown).

i] α1 expression in human dermal fibroblasts after 24 hours incubation in 10% FCS:



ii] α2 expression in human dermal fibroblasts after 24 hours incubation in 10% FCS:



iii] α5 expression in human dermal fibroblasts after 24 hours incubation in 10% FCS:

Fig. 5.1: Visualisation of intracellular and membrane-bound integrin subunits in primary human dermal fibroblasts

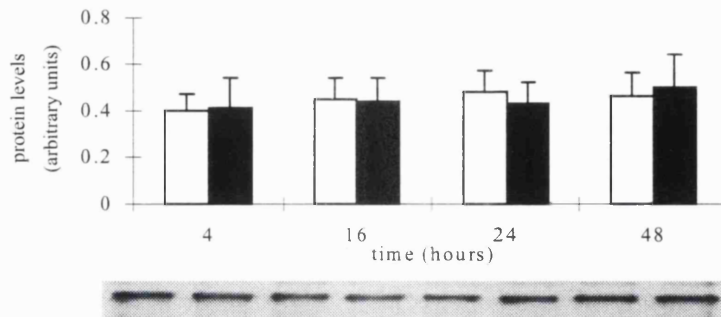
Figures 5.1i-iii show electron micrographs of α1, α2 and α5 integrin subunits in human dermal fibroblasts. Integrins were located using specific antibodies coupled to a FITC secondary. Control cultures using secondary antibody only showed no staining under identical conditions (not shown). All cultures were incubated in 10% serum for 24 hours prior to fixation and staining. All micrographs were compounded and taken at 640x magnification.

5.2.2 Mechanical load does not alter total expression of α1, α2, α5 or β1 integrin subunits

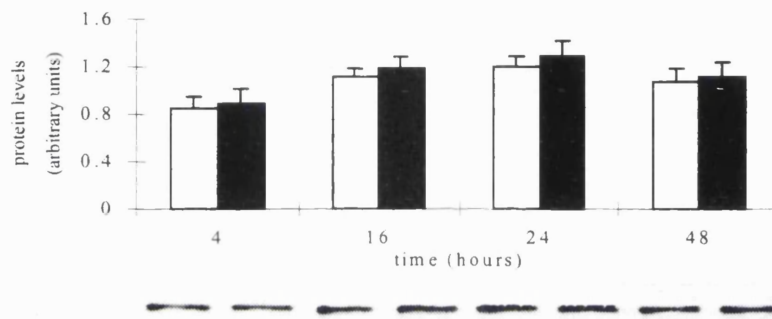
Figures 5.2 to 5.5 show the total expression (intracellular and membrane-bound) of the integrin subunits α1, α2, α5 and β1 in response to serum/TGFß and mechanical load. Fig 5.2a-c shows that cells incubated in serum-free media, 10% serum or TGFß respectively in the presence or absence of mechanical load did not exhibit any changes in total α1 integrin subunit expression. There were no significant changes in expression in any of the cultures at any time point investigated. Fig 5.3a-c shows the total expression of α2 integrin subunit under the same conditions. There were no significant changes in total α2 levels in serum-free media or in the presence of 10% FCS. TGFß stimulated α2 expression over the 48 hour time course of α2 integrin levels, however, this increase was not significant. Mechanical load had no additional effect on α2 integrin expression in any of the conditions investigated, at any time point.

Fig. 5.4a-c show the effects of serum-free media, 10% FCS or TGFß respectively, all with or without the application of mechanical load, on total α5 expression. As with both α1 and α2 subunits, α5 did not increase in response to growth factors over the 48-hour period. Load again had no effect. TGFß stimulated an increase in α5 expression over the time course, but this was not significant. β1 subunit expression is shown in Figs 5.5a-c in response to serum-free media, 10% FCS and TGFß respectively. Total levels of β1 were high in all cultures, but there were no significant alterations in expression in any of the conditions tested. Mechanical load had no additional effect.

5.2a: Serum-free



5.2b: 10% FCS



5.2c: TGFß

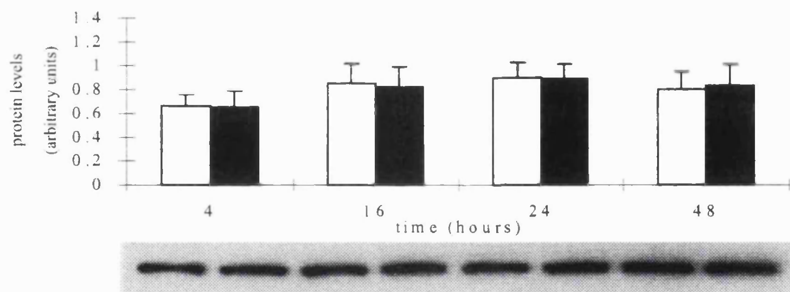
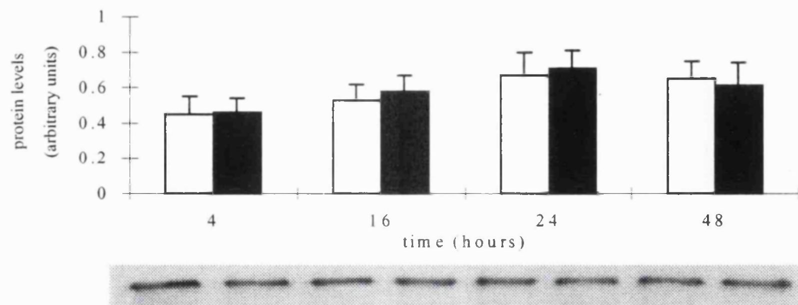


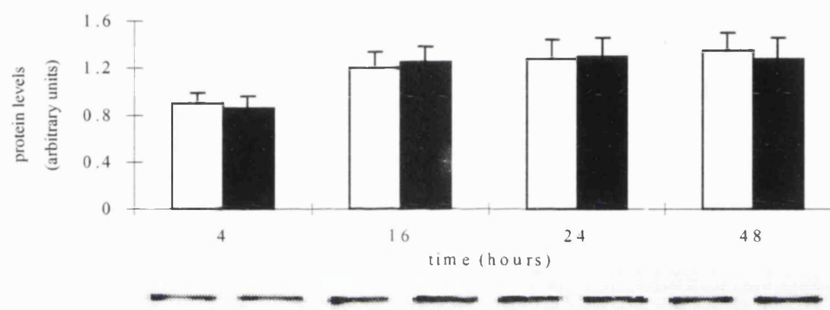
Fig. 5.2: Total α1 integrin subunit expression is not altered by mechanical load and serum or TGFβ:

Total integrin subunit expression was measured by immunoprecipitation using specific antibodies, followed by western blotting. Cells were left unloaded ( ) or mechanically loaded (•) in the presence of serum-free media (a), 10% FCS (b) or TGFß (c) for up to 48 hours, following which cultures were harvested for analysis. Immunoprecipitated protein levels were quantitated using densitometric scanning. 3 wells of fibroblasts were used for each measurement, and each experiment was repeated 3 times to gain an average.

5.3a: Serum-free



5.3b: 10% FCS



5.3c: TGFß

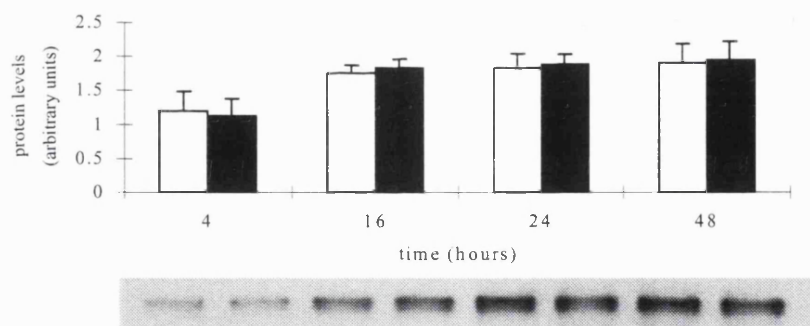
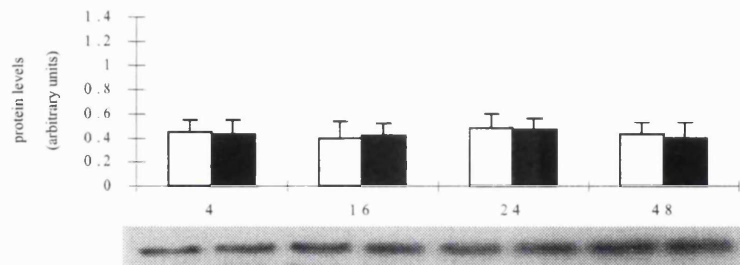


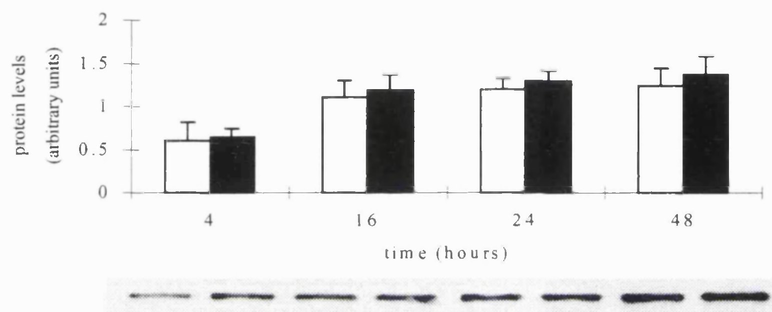
Fig. 5.3: Expression of α2 integrin is unaltered in the presence of serum/TGFβ and load

Total integrin subunit expression was measured by immunoprecipitation using specific antibodies, followed by western blotting. Cells were left unloaded ( ) or mechanically loaded (•) in the presence of serum-free media (a), 10% FCS (b) or TGFß (c) for up to 48 hours, following which cultures were harvested for analysis. Immunoprecipitated levels were quantitated using densitometric scanning. 3 wells of fibroblasts were used for each measurement, and each experiment was repeated 3 times to gain an average.

5.4a: Serum-free



5.4b: 10% FCS



5.4c: TGFß

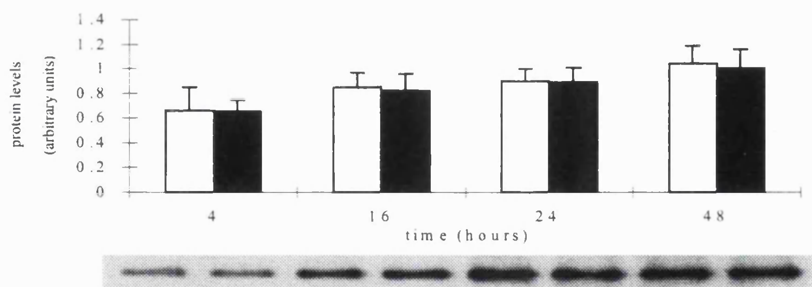
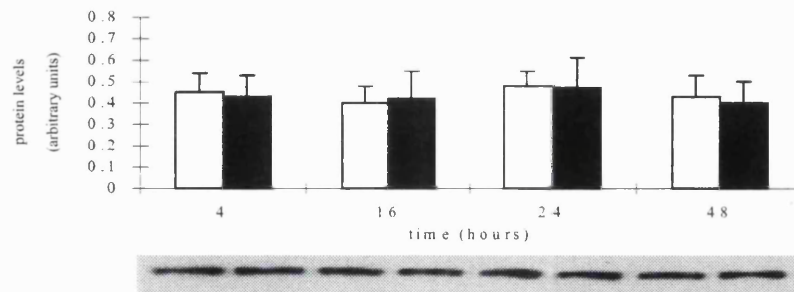


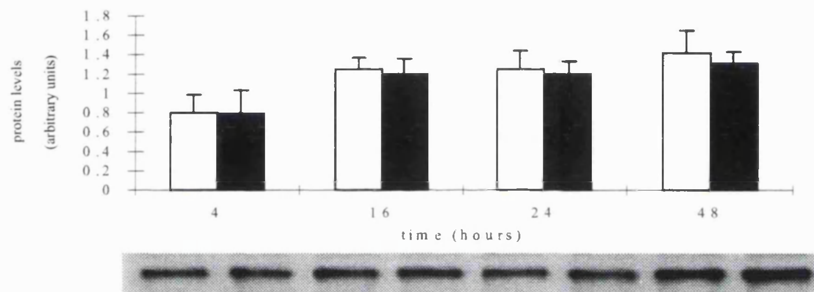
Fig. 5.4: Total expression of α5 is unaltered with serum or TGFß and load

Total integrin subunit expression was measured by immunoprecipitation using specific antibodies, followed by western blotting. Cells were left unloaded ( ) or mechanically loaded (•) in the presence of serum-free media (a), 10% FCS (b) or TGFß (c) for up to 48 hours, following which cultures were harvested for analysis. Immunoprecipitated levels were quantitated using densitometric scanning. 3 wells of fibroblasts were used for each measurement, and each experiment was repeated 3 times to gain an average.

5.5a: Serum-free



5.5b: 10% FCS



5.5c: TGFß

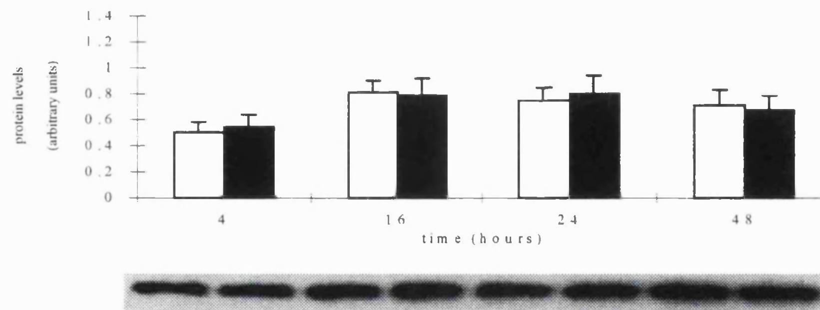


Fig. 5.5: Total expression of β1 integrin is unaltered in response to serum or TGFβ with load

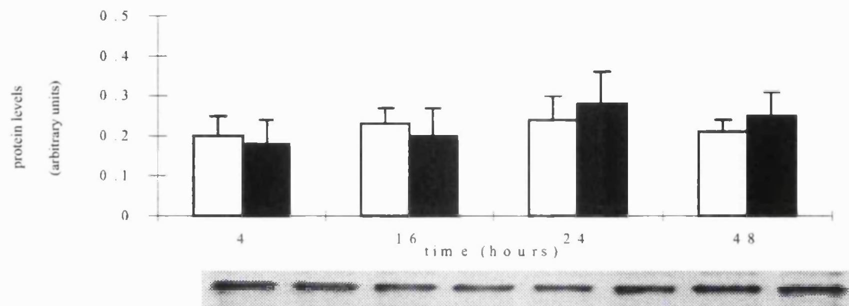
Total integrin subunit expression was measured by immunoprecipitation using specific antibodies, followed by western blotting. Cells were left unloaded ( ) or mechanically loaded (•) in the presence of serum-free media (a), 10% FCS (b) or TGFß (c) for up to 48 hours, following which cultures were harvested for analysis. Immunoprecipitated levels were quantitated using densitometric scanning. 3 wells of fibroblasts were used for each measurement, and each experiment was repeated 3 times to gain an average.

5.2.3 Mechanical load alters cell surface expression of α1 and α2 integrin subunits.

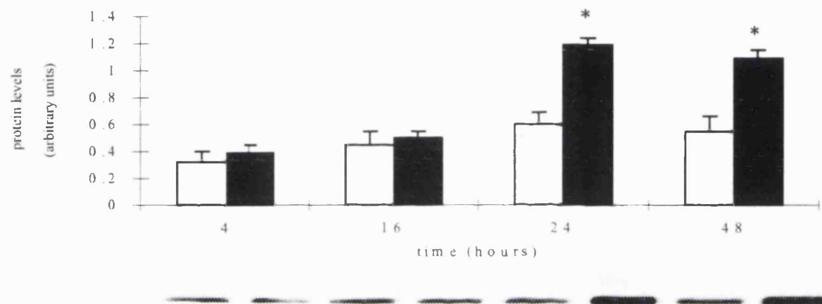
Figures 5.6 to 5.9 show the expression of the four integrin subunits, α1, α2, α5 and β1, on the cell-surface only, following incubation with serum or TGFß in the presence of mechanical load. Fig. 5.6a-c show α1 subunit expression at the cell surface, in the presence of serum-free media, 10% FCS or TGFß respectively. The data shows no increase in α1 cell surface expression in serum-free medium at any of the time points, with or without the application of load. In the presence of 10% FCS however, there was a 2-fold ($p<0.01$) increase in cell-surface α1 levels after both 24 and 48 hours in loaded versus control cultures. This increase was also seen in cultures treated with TGFß. α2 cell-surface expression in response to serum-free media, 10% FCS and TGFß, is shown in Fig 5.7a-c respectively. No change in membrane expression of this subunit was seen in serum-free medium at any time point with and without mechanical load. However, in the presence of 10% FCS or TGFß, levels of α2 at the cell surface are transiently increased by 2-fold ($p<0.01$) at 16 hours in loaded versus control cultures. Expression returned to control levels at the 24 hour time point.

Figs 5.8a-c show α5 cell-surface expression in response to serum-free media, 10% FCS or TGFß respectively. There was no significant difference between any of the time points investigated in terms of α5 expression in any of the conditions tested. Mechanical load had no effect at any time point. β1 subunit expression in response to serum-free media, 10% FCS or TGFß is shown in Fig 5.9a-c respectively. As with α5, no changes in cell surface expression were seen with any of the conditions tested, at any of the time points investigated.

5.6a: Serum-free



5.6b: 10% FCS



5.6c: TGFß

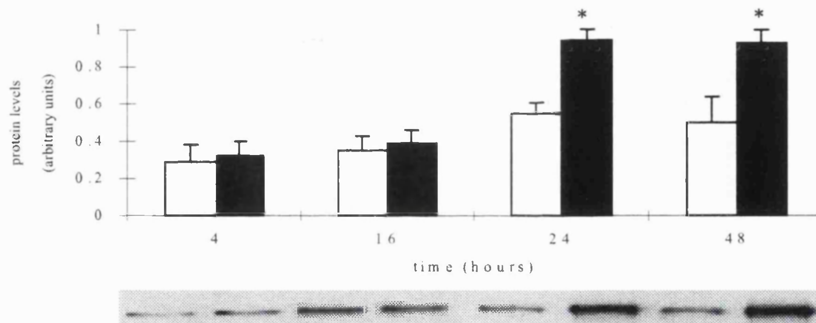
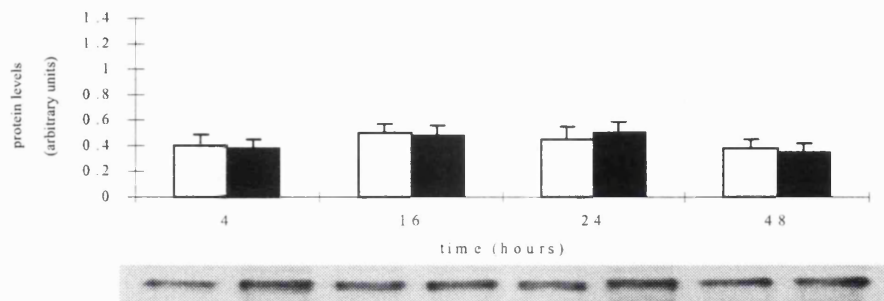


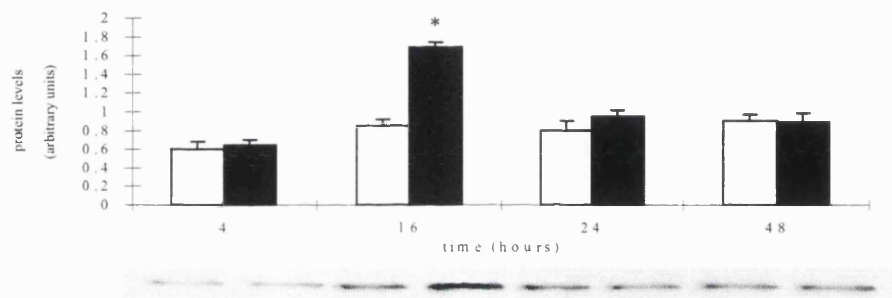
Fig. 5.6: Cell-surface levels of $\alpha 1$ integrin are altered in the presence of mechanical load and growth factors over 48 hours

Cell-surface integrin subunit expression was measured by biotinylation of membrane proteins and immunoprecipitation using specific antibodies, followed by western blotting. Cells were left unloaded ( ) or mechanically loaded (•) in serum-free media (a), 10% FCS (b) or TGFß (c) for 48 hours, and cultures were harvested for analysis. Levels were quantitated using densitometric scanning. 3 wells of fibroblasts were used for each measurement, and each experiment was repeated 3 times to gain an average.* denotes p<0.01 vs. control

5.7a: Serum-free



5.7b: 10% FCS



5.7c: TGFß

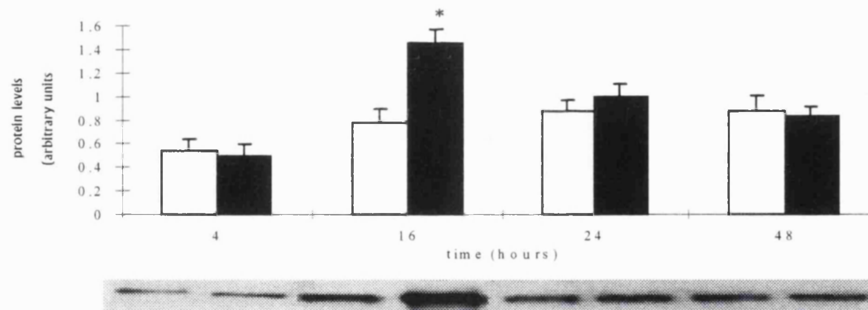
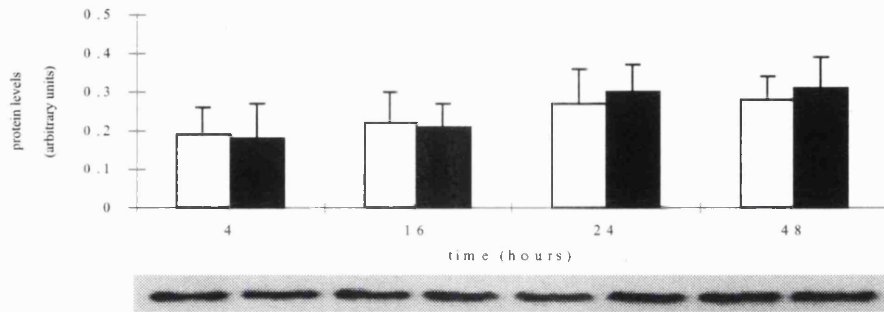


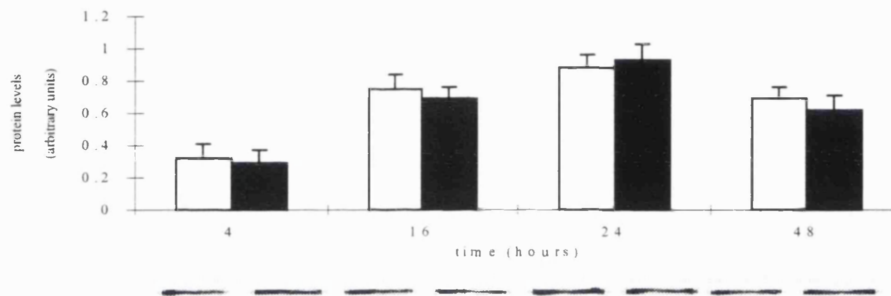
Fig. 5.7: Cell-surface levels of a2 integrin are transiently altered in the presence of mechanical load and growth factors over 48 hours

Cell-surface integrin subunit expression was measured by biotinylation of membrane proteins and immunoprecipitation using specific antibodies, followed by western blotting. Cells were left unloaded ( ) or loaded (•) in serum-free media (a), 10% FCS (b) or TGFß (c) for up to 48 hours, following which cultures were harvested for analysis. Levels were quantitated using densitometric scanning. 3 wells of fibroblasts were used for each measurement, and each experiment was repeated 3 times. *denotes $p<0.01$ vs. control.

5.8a: Serum-free



5.8b: 10% FCS



5.8c: TGFß

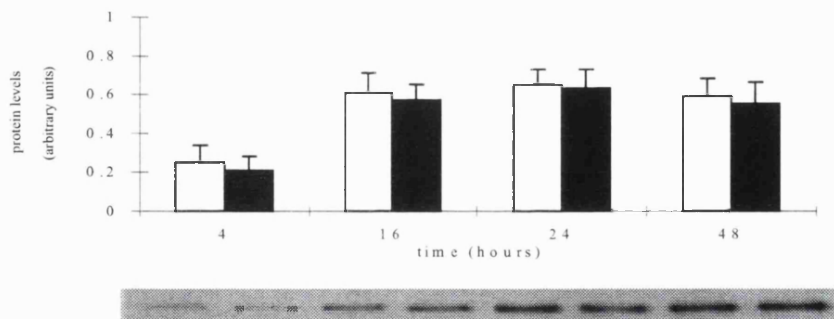
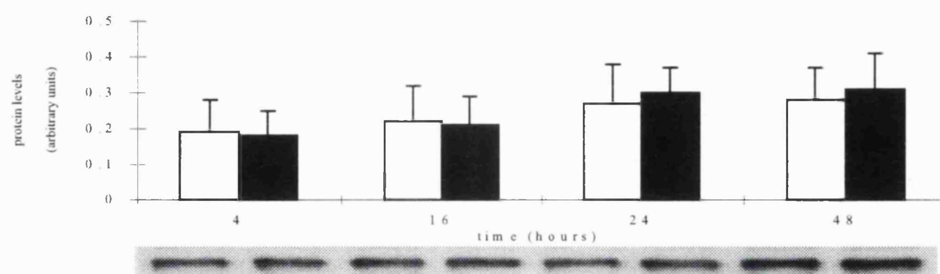


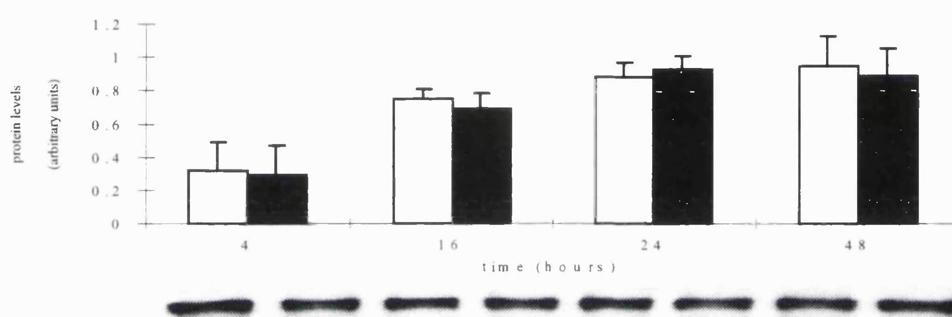
Fig. 5.8: Mechanical load and serum/TGFβ do not alter cell surface expression of a5 integrin

Cell-surface integrin subunit expression was measured by biotinylation of membrane proteins and immunoprecipitation using specific antibodies, followed by western blotting. Cells were left unloaded ( ) or loaded (•) in the presence of serum or TGFß for up to 48 hours, following which cultures were harvested for analysis. Immunoprecipitated levels were quantitated using densitometric scanning. 3 wells of fibroblasts were used for each measurement, and each experiment was repeated 3 times

5.9a: Serum-free



5.9b: 10% FCS



5.9c: TGFß

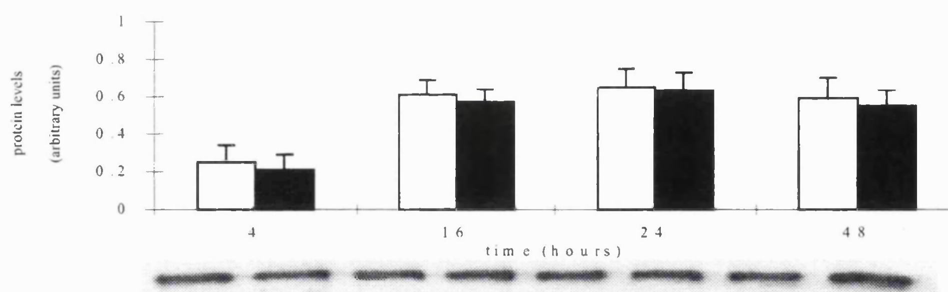


Fig. 5.9: Mechanical load and serum/TGFβ do not alter cell surface expression of β1 integrin

Cell-surface integrin subunit expression was measured by biotinylation of membrane proteins and immunoprecipitation using specific antibodies, followed by western blotting. Cells were left unloaded ( ) or loaded (•) in the presence of serum or TGFß for up to 48 hours, following which cultures were harvested for analysis. Immunoprecipitated levels were quantitated using densitometric scanning. 3 wells of fibroblasts were used for each measurement, and each experiment was repeated 3 times.

5.3 DISCUSSION

The regulation of integrin expression and function is of great importance in controlling the nature of cell-matrix interactions. Similarly, the effects of mechanical load on these processes is also important, and assists in an understanding of both the mechanisms of integrin action, and the changes in cell-matrix contact in the presence of load. However, both these areas are currently very poorly understood. The data presented in this chapter demonstrates that growth factors increase total levels of integrin subunits, but cell surface levels remain largely unchanged. Mechanical load alters the cell surface expression of specific integrins in the presence of serum or TGFß1.

The data demonstrates that both serum and TGFß1 upregulate total levels of α1, α2, α5 and β1 subunits. Data in Figure 5.2-5.5 shows that total levels of integrin protein were raised in cultures treated with 10% FCS or TGFß1, and cell surface levels were also increased compared to serum-free control levels. This reflects findings in lung fibroblasts in previous studies (Heino *et al*, 1989). As integrin mRNA levels were not assessed in response to growth factors or load in this thesis, it is not possible to say whether the effects seen are due to an increase in transcription, or translation of these receptor proteins. Indeed, past studies have not assisted in clarifying the site of action of TGFß1 in terms of integrin regulation. One report states that TGFß1 does not increase the level of α5 mRNA, but rather prevents decay of existing RNA (Roberts *et al*, 1988). A different study however, argues that steady-state levels of α5 and β1 mRNA both undergo a net increase in the presence of TGFß1 in comparison to levels of glyceraldehyde-3-phosphate dehydrogenase mRNA used as a control (Heino *et al*,

1989). The latter report also describes that the elevated rate of β1 subunit maturation seen in this system, from 10 hours to 3 hours is as a consequence of the higher levels of alpha subunits available for assembly of integrin complexes. However, it is possible that in addition to this effect, TGFß1 might directly control the mechanism for intracellular retention of unassembled subunits. TGFßs do increase the mRNA levels of multiple cell adhesion proteins and the available evidence suggests the involvement of transcriptional as well as post-transcriptional events in this response (Rossi *et al*, 1988; Dean *et al*, 1988).

TGFß, being one of the most potent stimulators of matrix synthesis, causes enhancement of type I collagen incorporation into the ECM surrounding fibroblasts (Ignotz and Massague, 1987). Two integrins, α1β1 and α2β1 are upregulated in response to TGFß1, and also mediate cell attachment to collagen I (Wayner and Carter, 1987). The involvement of the α5β1 fibronectin receptor in the enhancement of cellular adhesion to fibronectin in response to TGFß1 and TGFß2 has also been described (Ignotz and Massague, 1987). Again, the co-regulation of individual integrin subunits and ECM proteins by TGFß1 and other factors is still very poorly understood. However, evidence accumulated thus far would suggest that it is accomplished via overlapping but independent mechanisms. This highlights the importance of a number of different stimuli being very tightly coordinated to form the very ordered series of events required for correct signalling.

Two of the four receptor subunits analysed in this chapter, α1 and α2, exhibited increased recruitment at the cell membrane in response to load, whereas α5 and β1 did not. The specificity of response to load and also the timing are both very interesting. The α1 subunit was increased at the cell surface after 24 hours of cyclical

load, compared to unloaded controls, and these levels were sustained for the following 24 hours of culture. However, the α2 subunit was expressed at higher levels after 16 hours in loaded versus control cultures, and then returned to control levels by the 24 and 48 hours time points. This transient recruitment of α2 to the cell surface is very interesting and has not been previously reported in the context of load responses or indeed growth factor responses. The recruitment of β1 integrins was not seen in response to load in the data presented in this chapter. This finding is in agreement with a recent study in endothelial cells, which shows no change in β1 expression in response to cyclical mechanical load over 24 hours. This report has also demonstrated no change in total or cell surface α2 or α5 integrin subunit (Yano *et al*, 1997). However, changes in organisation on the cell surface into linear patterns were reported for β1, α5 and α2 subunits, and increase in the phosphorylation of focal adhesion proteins were also observed (Yano *et al*, 1997). Therefore, in this study, despite exhibiting no change in expression of integrins in response to load, it would appear that both α2β1 and α5β1 may be responsive to stretch merely through the reorganisation and subsequent increased signalling to the cell nucleus.

Another report has demonstrated an upregulation of β3 integrin subunit in endothelial cells at both mRNA and protein levels in response to uniaxial cyclical load (Suzuki *et al*, 1997). Again, the subunit was reorganised within the focal adhesion sites, conferring an increased capacity to respond to external signals. Interestingly, the effects of mechanical load on integrin expression also apply to bone cells. One report indicates that in osteosarcoma cells, β1 integrin mRNA is increased in response to cyclical stretch, and that this subunit is reorganised and distributed at the cell surface after just 4 hours (Carvalho *et al*, 1995). It would seem, therefore, that these changes

in receptor expression are probably not specific to one cell type, and rather may represent a more generalised cell response to mechanical stimulation. Indeed, there are reports investigating shear stress, responses in endothelial cells, which support this theory very well. One report demonstrated an induction in integrin $\alpha5\beta1$ expression in bovine endothelial cells after 24 hours of shear stress (Girard *et al*, 1995). Cells and integrins are also reorganised following stretch at a very early time point, and the cell alters in morphology from polygonal shape to spindle-like shape, of which long axis is aligned to the stretch axis (Ando et al, 1994; Girard *et al*, 1995). In all these studies, cell morphology was altered up to 1 hour after the onset of stretch, followed at a later time point by changes in integrin expression (6-24 hours). This would suggest that change in integrin organisation and cellular morphology is involved in much earlier load-induced signalling events. Transcriptional and cell-surface changes in integrin receptor expression may be more involved in the later signalling stages, which dictate a biochemical response to stretch.

It may well be the case that mechanical load is affecting the normal integrin cycling between the cell membrane and internalisation/degradation. It has been reported previously that integrin binding to the extracellular matrix interrupts the normal integrin metabolic cycle, which permits internalisation of cell surface receptors. Occupation of integrins at the cell surface instead permits the retention of integrins at the cell surface and subsequently increases the cellular capacity to signal through this pathway (Dalton *et al*, 1995). This may well be the case with cyclical mechanical load. Changes in cell surface expression may be due to the constant stimulation of focal adhesion clusters at the cell membrane as the cell is stretched. This would then lead to recruitment of specific integrins to these clusters, as the normal metabolic cycling is interrupted by the application of load. The increase in integrin levels at the

cell surface, indeed in some cases transiently, may be also due to an increased requirement for adhesion proteins to prevent cell detachment under such strain. The specificity of this integrin recruitment response may also be matrix-dependent, as has been shown in other systems. In this system, it would seem that the collagen receptors are the most important integrins to be affected and altered in the presence of load. This is logical in a system where the fibroblasts are both plated on collagen, and are synthesising and depositing excessive collagen in response to mechanical load.

The data presented in this chapter therefore has demonstrated that, as in other systems, mechanical load alters cell surface levels of $\alpha 1$ and $\alpha 2$ integrin subunits, whilst total levels remain unaffected. The functional role of these changes in integrin recruitment to the cell membrane in mediating a load-induced increase in collagen synthesis must now be assessed. These studies will ascertain if integrins are involved in mechanotransduction from a mechanical to a biochemical signal in this system.

CHAPTER SIX

Results:

Integrin regulation of load-induced collagen deposition

6.1 INTRODUCTION

Integrins, as described in the previous chapter, mediate a variety of signalling cascades and may transduce mechanical stimuli into biochemical signals. It can be hypothesised that the transduction of a mechanical signal to the nucleus via integrins may be responsible for controlling biological responses to stretch such as matrix synthesis and cell cycle progression. Whilst there have been a number of studies addressing the integrin-dependent signalling events in response to stretch, very few of the reports investigate the functional role of the integrin-mediated signals in new protein synthesis.

The role of integrins in the transduction of signals has been investigated in many different capacities. Integrin knockout animals or cells have been used in the study of integrin function (Gardner *et al*, 1995;). However as the majority of integrin knockout animals are not viable, alternative methods have been employed to interfere or inhibit the binding and signalling capabilities of the ECM receptors. As integrins are heterodimeric molecules, different sites on the subunits are inhibited when using blocking agents. Many peptides have been designed against specific sequences of both α2 and α5 integrin subunits (Humphries *et al*, 1997) and have proven to have very different properties depending upon the part of the protein that is targeted. Antibodies have been commonly used to inhibit or stimulate different integrin functions by inducing a conformational change in the receptor to modulate the affinity/avidity for the ligands, and have been used in inhibition of adhesion, signalling and cell proliferation/cell cycle events.

The use of antisense oligonucleotides in these inhibition studies is still rare, as the technology is still being developed. Also, as very little is known about integrin metabolism and cycling from intracellular to cell membrane, antisense can be a difficult technique to apply. However, a few studies have now begun to use this technology in the successful blocking of both adhesion and function (Orci et al, 1998). Integrin chimeras and truncated structures have also been used in many studies to investigate the properties of different parts of integrin subunits in various signalling and matrix attachment events. This technique involves combination subunits, which comprise a cytoplasmic sequence from one integrin combined with the extracellular domain of another (Kern and Marcantonio, 1998; Assoian, 1997). This allows sequences within specific subunits to be separated and the functional importance assessed.

All of the above techniques have been used in studies elucidating the role of specific integrins on collagen synthesis. Both $\alpha1\beta1$ and $\alpha2\beta1$ are expressed in abundance on mesenchymal cells (Voight *et al*, 1995) and are primarily collagen-binding integrins. The involvement of these receptors in regulating collagen synthesis has been well studied. Unusually, $\alpha1$ null mutant animals show no gross developmental deficits, but null fibroblasts *in vitro* show a deficiency in attachment to collagen IV and a deficiency in collagen dependent proliferation (Gardner *et al*, 1996; Pozzi *et al*, 1998). However, only cells deficient in $\alpha2$ or $\alpha3$ display any deficit in binding collagen I demonstrating the specificity of function between the different collagen-binding integrins.

Several lines of evidence have suggested regulatory roles for $\alpha1\beta1$ and $\alpha2\beta1$. In osteosarcoma cells, the level of $\alpha2\beta1$ integrin correlates with the expression of native

collagenase (MMP1) (Shingleton *et al*, 1996) whereas α1β1 seems to be responsible for the downregulation of collagen mRNA levels seen when the cells are suspended in collagen gels (Langholtz *et al*, 1995). The use of specific anti-integrin antibodies supports a role for α1β1 as a regulator of collagen synthesis in that an activating anti-α1 antibody accentuates the normal downregulation of fibroblast collagen expression in contracted collagen gels (Langholz *et al*, 1995). It has also been shown that skin fibroblasts derived from patients with scleroderma, a fibrotic skin disorder, show a relative decrease in the expression of α1β1 and thus display uncontrolled collagen synthesis (Ivarsson *et al*, 1993). This supports the role of α1β1 integrin in the regulation of collagen production, and suggests further that this mechanism may be of importance in fibrosis or excessive scarring disorders.

α2β1 has also been widely studied in the control of collagen synthesis, and also in the regulation of contraction of collagen gels in vitro. Studies using antibodies blocking α2 and β1 have shown that this interferes with gel contraction, and it was proposed that α2β1 represented the receptor responsible for this contraction process (Klein *et al*, 1991). It is also known that α2β1 can bind to F actin and therefore has a direct mechanical link to the cyotskeleton (Kieffer *et al*, 1995). In terms of regulation of collagen synthesis, α2β1 has been studied in the context of feedback regulation by binding to specific sequences from the cleaved procollagen c-terminal propeptide. This collagen receptor was identified to be the cell surface receptor for the c-terminal peptide as well as recognising the triple helical regions of the intact procollagen molecule (Weston *et al*, 1994). Conflicting reports have identified different peptides within the c-terminal peptide that both inhibit and stimulate collagen synthesis (Aycock *et al*, 1986; Katayama *et al*, 1993). It would appear that the nature of the

sequence is very important in the feedback regulation of procollagen synthesis, and controversy still exists as to whether this is at a transcriptional or post-transcriptional level (Bhattachatyya-Pakrasi *et al*, 1998). However, $\alpha2\beta1$ would still appear to be very important in both mechanical and biochemical processes of regulating collagen synthesis and organisation.

As briefly mentioned in section 1.5.5 of the general introduction, integrins have been widely implicated in stretch-induced signalling cascades. One of the most comprehensive studies analysed the effects of load on members of the MAPK family; extracellular regulated kinase (ERK), p38 and c-Jun levels, in cardiac fibroblasts. This study demonstrated that $\alpha4\beta1$ and an RGD-directed non- $\alpha5\beta1$ integrin activated ERK2 in response to mechanical stimulation, causing increased phosphorylation and overall increased levels. Activation of Jnk by load could not be blocked with inhibitors suggesting that an RGD-independent integrin or non- $\alpha4\beta1$ integrins were involved. Both ERK2 and Jnk were only activated by load if the cells were plated onto fibronectin (MacKenna *et al*, 1998).

Integrins have also been investigated in endothelial cell responses to shear stress and cyclical mechanical load. Integrin $\alpha v\beta3$ is involved in the recruitment and subsequent signalling of Shc in response to shear stress. However, cyclical load stimulates both morphological changes and increased tyrosine phosphorylation in HUVECs, but neither of these effects are mediated by $\alpha5$, $\alpha2$ or $\beta1$ integrins as demonstrated by the use of blocking antibodies (Yano *et al*, 1997). Stretch-induced proliferation of smooth muscle cells has been shown to be dependent on $\alpha5\beta1$ integrin, and therefore inhibited by both blocking antibodies and soluble fibronectin (Wilson *et al*, 1997). Therefore, the direct role of integrins in both signalling and biochemical responses to

load is still very much under investigation. However, there is definite evidence that these cell-matrix receptors are involved in mechanotransduction in many cell types.

Aims

The aim of this chapter is to assess the role of $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha5\beta1$ integrins in the regulation of collagen synthesis and PCP expression in response to load. It has been shown in the last chapter that both $\alpha1$ and $\alpha2$ integrin subunits alter in cell surface expression in response to load, and indeed both of these integrins are already implicated in collagen synthesis regulation by different mechanisms. The study aims to investigate the roles of $\alpha1$, $\alpha2$ and $\alpha5$ in processes leading to excessive ECM deposition in response to load. The role of these integrins in collagen synthesis and processing has been assessed using integrin function blocking antibodies and antisense oligonucleotides.

6.2 RESULTS

6.2.1 α1β1 is involved in procollagen synthesis in response to growth factors and load

Fig. 6.1 and 6.2 show the effects of blocking α1β1 on serum, TGFß and mechanical load-induced procollagen synthesis and insoluble collagen deposition respectively after 48 hours incubation. Total procollagen synthesis was significantly reduced in both loaded and control cultures incubated with α1β1 integrin blocking antibody. Control cultures containing blocking antibody showed an inhibition in total procollagen synthesis to 50±5% ($p<0.01$) of serum control levels. Mechanically loaded cultures in the presence of blocking antibody showed a decrease in procollagen synthesis levels to 35±7% of loaded serum controls. This inhibition reduced levels of procollagen synthesis in mechanically loaded cultures to that in unloaded cultures containing blocking antibody. These effects were reproduced in serum-free cultures supplemented with TGFß. Inhibition of procollagen synthesis by antibodies in control and loaded cultures reduced levels to 48±6% and 35±4% ($p<0.01$) respectively compared with levels in control cultures.

Insoluble collagen deposition was measured in identical cultures to those described above. Insoluble collagen deposition in both serum and TGFß antibody-treated cultures remained significantly higher than unloaded controls ($p<0.01$). Control unloaded cultures also did not show any changes in collagen deposition in the presence of the α1β1 functional blocking antibody.

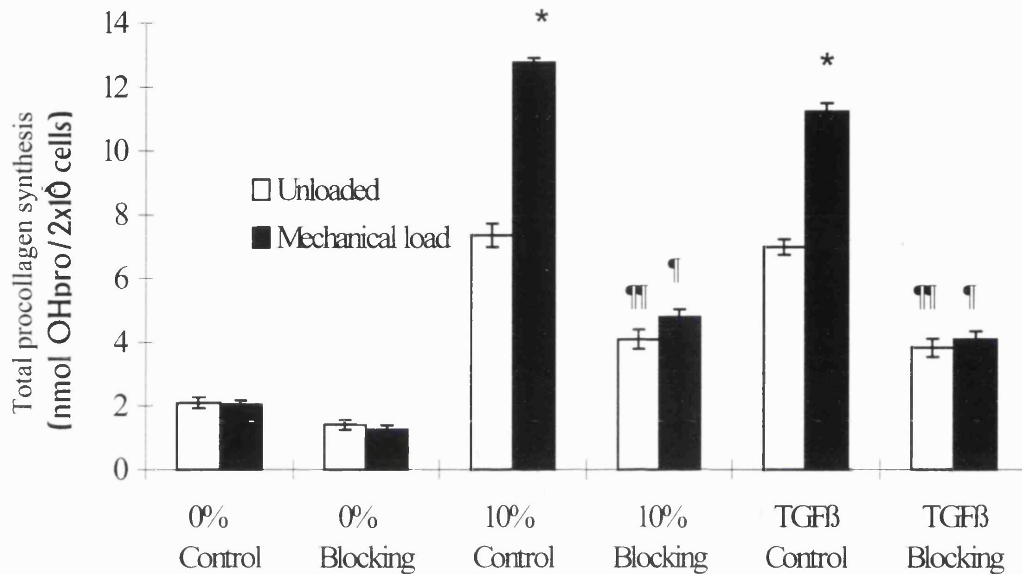


Fig. 6.1: $\alpha 1\beta 1$ is involved in procollagen synthesis in response to growth factors:

Figure showing total procollagen synthesis after 48 hours incubation in the presence of serum or TGFß1 with and without the application of mechanical load. Blocking antibodies to $\alpha 1\beta 1$ integrin were added at 10ug/ml, the maximum concentration before cells began to detach from the plate, 30 minutes prior to the start of the experiment. Procollagen synthesis is represented as nmol OHpro/$2\text{x}10^5$ cells. Experiments were repeated 3 times, an average of 6 wells used in each experiment to gain a mean. * denotes $p<0.001$ level of significance compared with unloaded cultures. ¶ and ¶¶ denotes $p<0.001$ and $p<0.01$ level of significance of blocking compared with respective control cultures.

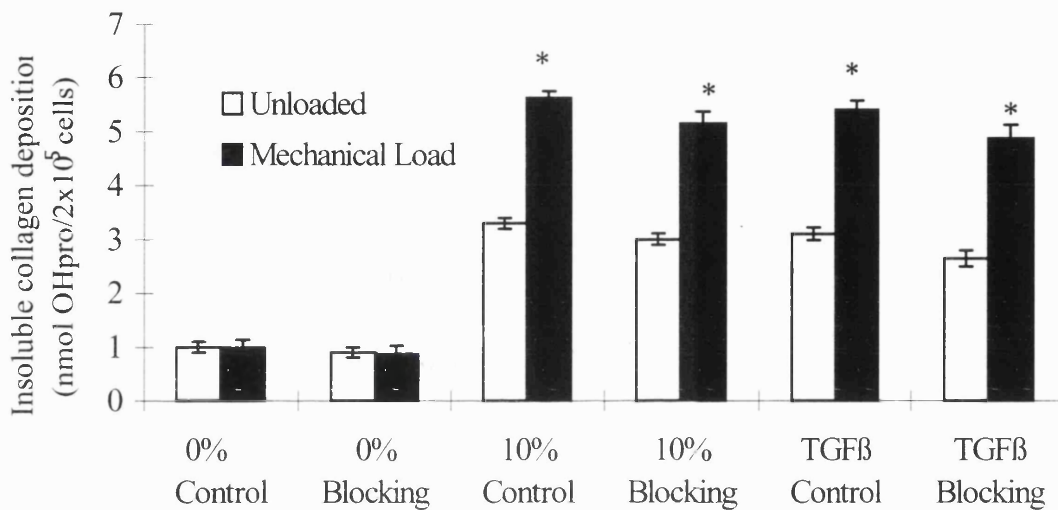


Fig. 6.2: α1β1 is not involved in insoluble collagen deposition in response to growth factors or load:

Figure showing insoluble collagen deposition (insoluble cell layer fraction) after 48 hours incubation in the presence of serum or TGFß1 with and without the application of mechanical load. Blocking antibodies to α1β1 integrin were added at 10ug/ml, the maximum concentration before cells begin to detach from the plate, 30 minutes prior to the start of the experiment. Collagen deposition is represented as nmol OHpro/2x10^5 cells. Experiments were repeated 3 times, an average of 6 wells used in each experiment to gain a mean. * and ** denote $p<0.001$ and $p<0.01$ level of significance respectively compared to unloaded control.

6.2.2 $\alpha 2\beta 1$ is involved in procollagen synthesis and collagen deposition in response to growth factors and load

The data shown in Figs 6.3 and 6.4 indicate the effects of $\alpha 2\beta 1$ integrin-blocking antibodies on total procollagen synthesis and insoluble collagen deposition respectively. Procollagen synthesis was unchanged in the presence of $\alpha 2\beta 1$ blocking antibody in all cultures containing serum-free media. Total procollagen synthesis was unaffected in unloaded control cultures in the presence of serum or TGFß and $\alpha 2\beta 1$ blocking antibody. In loaded cultures with both serum and TGFß, however, $\alpha 2\beta 1$ blocking antibody inhibited load-induced procollagen synthesis by 22±4% and 18±2% respectively ($p<0.05$). However, despite this reduction in procollagen synthesis, levels in mechanically loaded cultures were still significantly higher than levels in control cultures ($p<0.01$).

Collagen deposition was assessed in cultures incubated with serum or TGFß (Fig 6.4) in the presence of mechanical load. The effects of blocking antibodies to $\alpha 2\beta 1$ on collagen deposition was assessed after 48 hours. Blocking antibodies had no effect on collagen deposition in cultures incubated in serum-free media. Collagen deposition in control cultures was unaffected in the presence of blocking antibodies. Mechanically loaded samples in the presence of 10% serum showed a 46±5% inhibition of collagen deposition in the presence of $\alpha 2\beta 1$ blocking antibodies, thereby totally blocking the stretch-induced increase in collagen deposition. In TGFß treated cultures in the presence of mechanical load, the blocking antibody caused inhibition of deposition of insoluble collagen by 53±4%, reducing deposition to control levels.

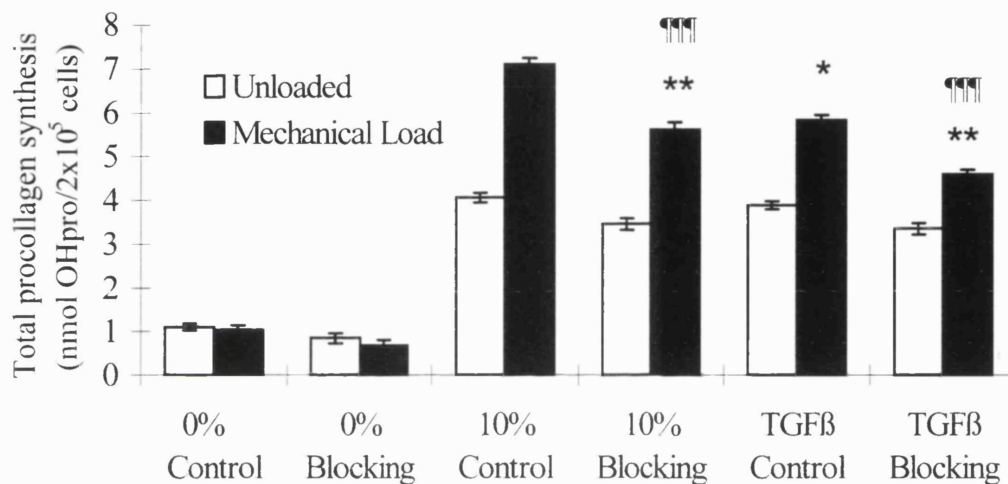


Fig. 6.3: α2β1 is involved in procollagen synthesis in response to growth factors and load:

Figure showing total procollagen synthesis after 48 hours incubation in the presence of serum or TGFß1 with and without the application of mechanical load. Blocking antibodies to α2β1 integrin were added at 8ug/ml, the maximum concentration before cells begin to detach from the plate, 30 minutes prior to the start of the experiment. Procollagen synthesis is represented as nmol OHpro/2x10^5 cells. Experiments were repeated 3 times, an average of 6 wells used in each experiment to gain a mean. * and ** denote $p<0.001$ and $p<0.01$ level of significance respectively compared to unloaded cultures. ¶¶¶ denotes $p<0.05$ level of significance in blocking vs. control cultures.

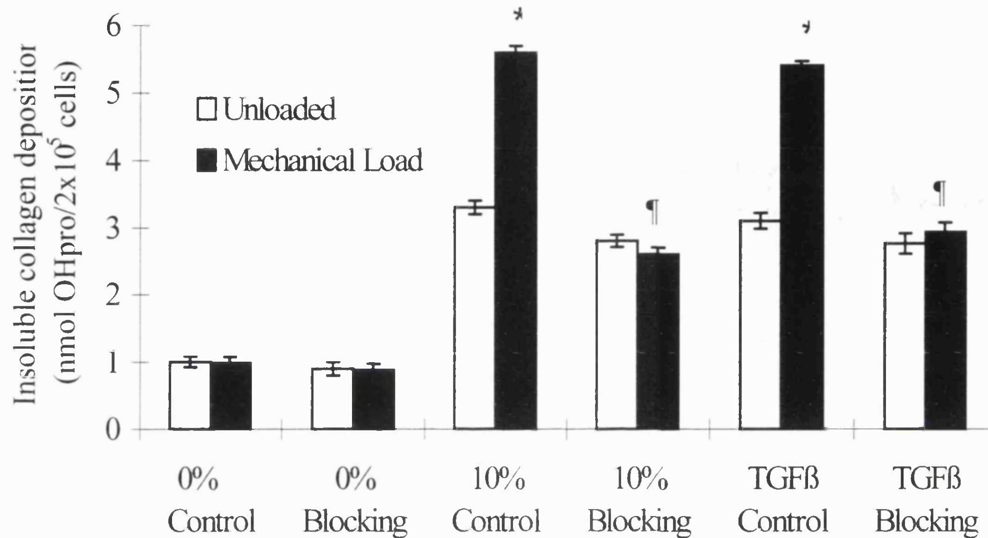


Fig. 6.4: α2β1 is involved in insoluble collagen deposition in response to growth factors and load:

Figure showing insoluble collagen deposition (insoluble cell layer fraction) after 48 hours incubation in the presence of serum or TGFß1 with and without the application of mechanical load. Blocking antibodies to α2β1 integrin were added at 8ug/ml, the maximum concentration before cells begin to detach from the plate, 30 minutes prior to the start of the experiment. Collagen deposition is represented as nmol OHpro/2x10^5 cells. Experiments were repeated 3 times, an average of 6 wells used in each experiment to gain a mean. * denotes $p<0.001$ level of significance in loaded compared to unloaded cultures. ¶ denotes $p<0.001$ level of significance in blocked vs. control cultures.

6.2.3 α5β1 is not involved in procollagen synthesis or deposition in response to growth factors or load

The data shown in Figs 6.5 and 6.6 indicate the effects of α5β1 integrin blocking antibodies on total procollagen synthesis and insoluble collagen deposition.

The total procollagen synthesis in cultures treated with 10% FCS and TGFß are shown in Fig. 6.5. Cultures incubated in serum-free media did not change procollagen synthesis levels in the presence of α5β1 blocking antibody, with or without mechanical load. Procollagen synthesis in serum and TGFß treated cultures was also unaffected by the presence of the α5β1 blocking antibody, both with or without mechanical loading.

Collagen deposition was assessed in cultures incubated with serum/TGFß in the presence of mechanical load, and the effects of addition of functional blocking antibodies was assessed after 48 hours. Data in Fig. 6.6 indicates that in all cultures, serum-free, 10% FCS and TGFß-treated, blocking antibodies to α5β1 integrin had no effect on insoluble collagen deposition. This was also the case in mechanically loaded cultures treated with serum or TGFß.

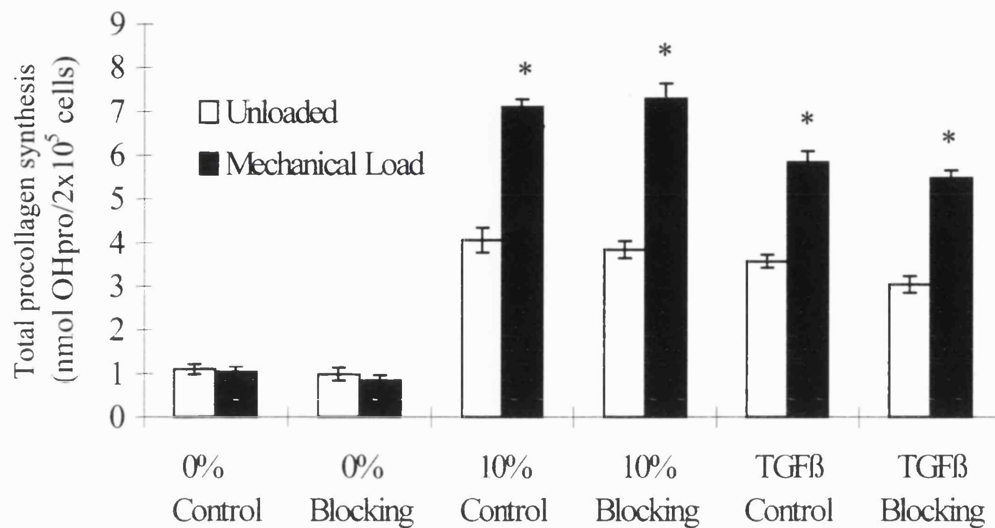


Fig. 6.5: α5β1 is not involved in procollagen synthesis in response to growth factors or load:

Figure showing total procollagen synthesis after 48 hours incubation in the presence of serum or TGFß1 with and without the application of mechanical load. Blocking antibodies to α5β1 integrin were added at 20ug/ml, the maximum concentration before cells begin to detach from the plate, 30 minutes prior to the start of the experiment. Procollagen synthesis is represented as nmol OHpro/2x10^5 cells. Experiments were repeated 3 times, an average of 6 wells used in each experiment to gain a mean. * denotes $p<0.001$ level of significance compared to unloaded cultures.

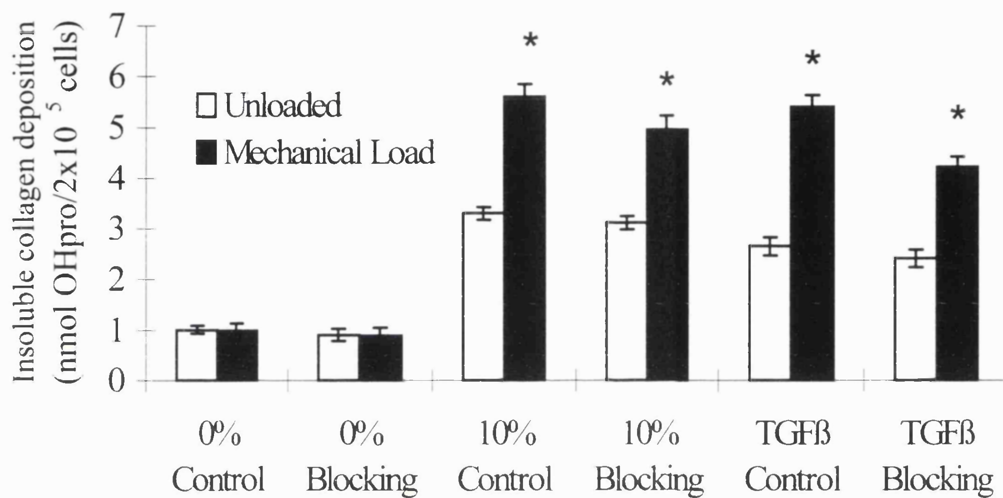


Fig. 6.6: α5β1 is not involved in insoluble collagen deposition in response to growth factors or load:

Figure showing insoluble collagen deposition (insoluble cell layer fraction) after 48 hours incubation in the presence of serum or TGFß1 with and without the application of mechanical load. Blocking antibodies to α5β1 integrin were added at 20ug/ml, the maximum concentration before cells begin to detach from the plate, 30 minutes prior to the start of the experiment. Collagen deposition is represented as nmol OHpro/2x10^5 cells. Experiments were repeated 3 times, an average of 6 wells used in each experiment to gain a mean. * denotes $p<0.001$ level of significance compared to unloaded cultures.

6.2.4 α2β1 blocking antibodies inhibit load-induced levels of PCP

The following data in Figs 6.7-6.9 indicate that integrins are also important in the regulation of levels of procollagen c-proteinase (PCP), the critical enzyme involved in collagen deposition, as previously described in Chapter 4.

Fig.6.7 shows the expression of PCP and PCPE protein in these cultures, and in identical cultures incubated with a function blocking antibody to α1β1 integrin. As previously described in Chapter 4, mechanical load stimulated a significant increase in PCP expression after 24 hours incubation both with serum and TGFß. PCPE protein levels were unaffected at any time point. Western blots indicate that the presence of the antibody did not alter the expression of PCP in unloaded or loaded cultures, both in the presence of serum or TGFß. PCPE levels were also unaffected by the blocking antibody.

Figure 6.8 demonstrates the effect of an α2β1 blocking antibody on PCP and PCPE protein expression. The increased PCP expression at 24 hours in control (non-specific IgG-treated) loaded cultures is blocked in parallel cultures treated with the specific α2β1 blocking antibody and serum or TGFß. PCPE protein levels were unchanged by the control or specific blocking antibody in all cultures.

Figure 6.9 shows the effects of α5β1 blocking antibody on PCP and PCPE protein levels in these cultures. Western blots indicate that the α5β1 blocking antibody did not have any effect on PCP or PCPE levels in cultures containing TGFß or 10% FCS. There was also no change in mechanically loaded cultures, compared to respective controls.

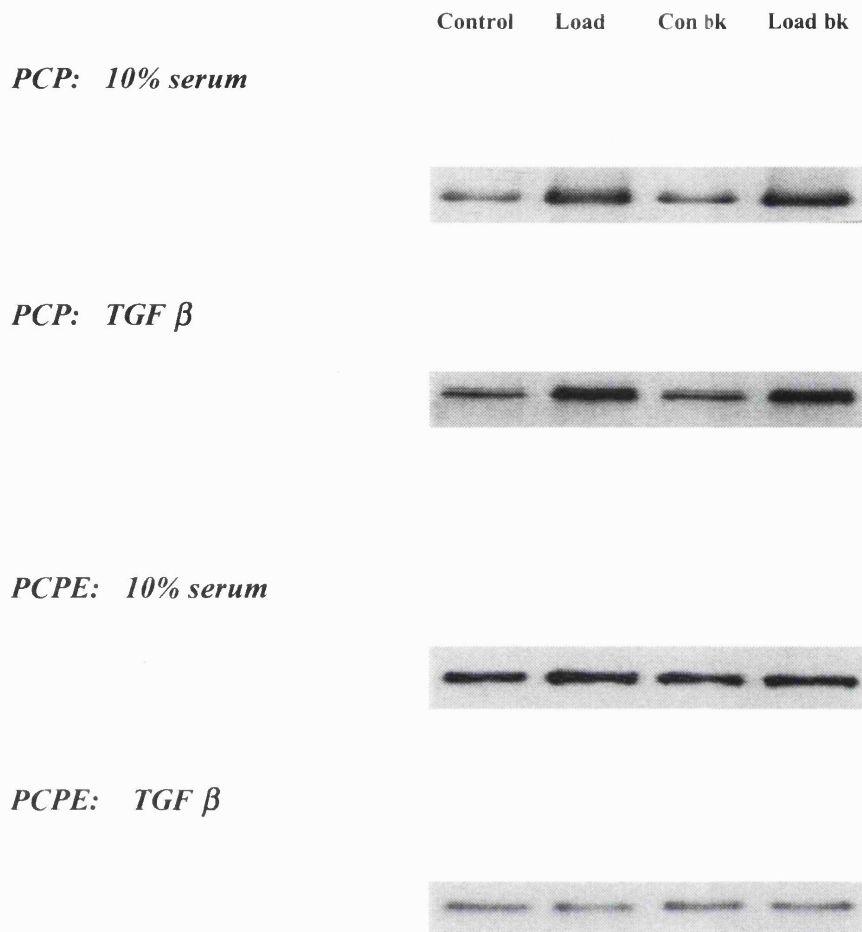


Fig 6.7: α1β1 has no effect on load-induced levels of PCP:

Western blots showing PCP and PCPE protein levels following 24 hours of incubation with serum or TGFß1 with (load) and without (control/con) mechanical load. α1β1 blocking antibodies (bk) were added into cultures 30 minutes prior to the start of the experiment at 10ug/ml, the highest concentration that did not induce cell detachment. 30ug of total protein was used per well and identical loading was confirmed by staining blots using ponceaus solution. Each experiment was repeated 3 times.

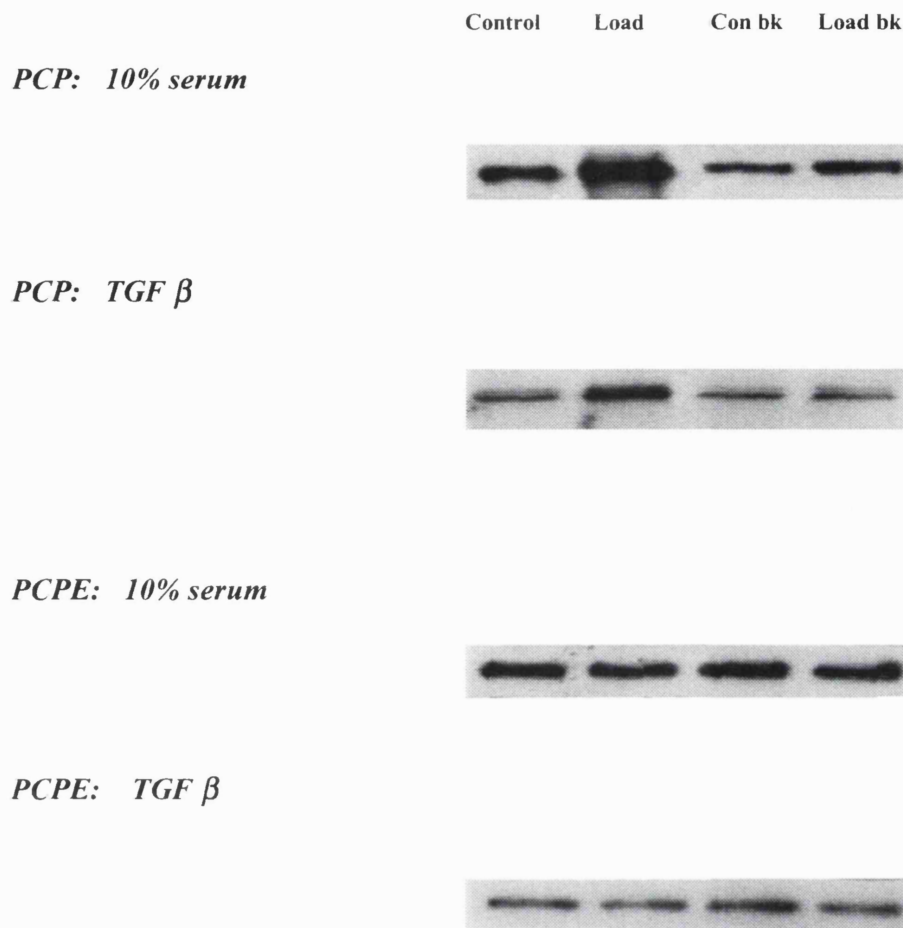


Fig 6.8: α2β1 regulates load-induced levels of PCP:

Western blots showing PCP and PCPE protein levels following 24 hours of incubation with serum or TGFß1 with (load) and without (control/con) mechanical load. α2β1 blocking antibodies (bk) were added into cultures 30 minutes prior to the start of the experiment at 8ug/ml, the highest concentration that did not induce cell detachment. 30ug of total protein was used per well and identical loading was confirmed by staining blots using ponceaus solution. Each experiment was repeated 3 times.

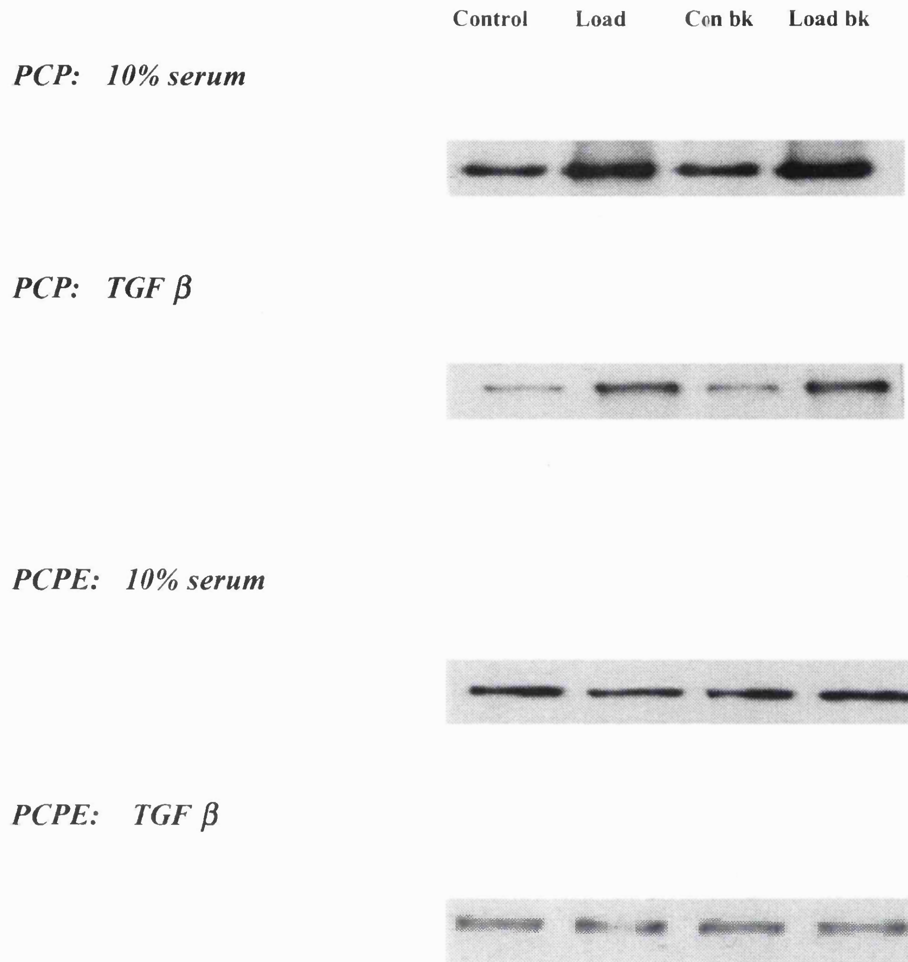


Fig 6.9: α5β1 has no effect on load-induced levels of PCP:

Western blots showing PCP and PCPE protein levels following 24 hours of incubation with serum or TGFβ1 with (load) and without (control/con) mechanical load. α5β1 blocking antibodies (bk) were added into cultures 30 minutes prior to the start of the experiment at 20ul/ml, the highest concentration that did not induce cell detachment. 30ug of total protein was used per well and identical loading was confirmed by staining blots using ponceaus solution. Each experiment was repeated 3 times.

6.2.5 Delivery of fluorescence-tagged antisense into human dermal fibroblasts using naked DNA

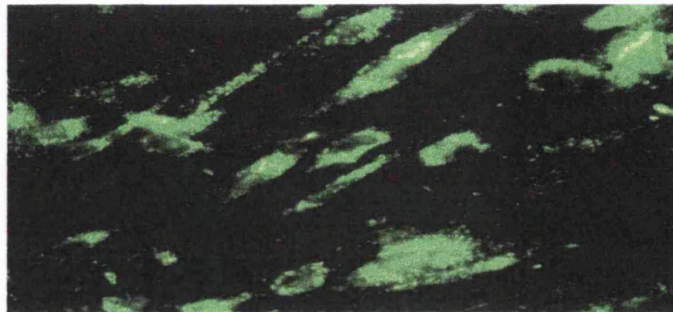
Figures 6.10 show delivery of FITC-tagged α2 integrin antisense oligonucleotide to human dermal fibroblasts over a time course of 72 hours. Fluorescence-tagged oligonucleotides were used prior to other antisense experiments to visually ensure that antisense was delivered into cells during the 72 hour period. The antisense was delivered to the cells at an optimal concentration of 2μM contained in serum-free media for 24 hours. This solution was then replaced with fresh media containing 10% FCS for the rest of the duration of the experiment. Cells were fixed at 30 minutes, 2, 4, 8, 16, 24, 48 and 72 hours following addition of antisense to the cells (time 0).

The confocal micrographs indicate that antisense oligonucleotide is not present within the cells by 30 minutes post-delivery. However, by 8 hours, all cells in each well assessed contained the FITC-tagged antisense, and this expression continued until 48 and 72 hours after the first delivery of antisense.

Table 6.1 shows lactate dehydrogenase (LDH) assay on cells incubated with antisense oligonucleotide for up to 72 hours. Data indicates that there was no significant change in cell viability in control cultures compared to antisense-treated cultures at any time point. Cells did not alter in morphology upon addition of the oligonucleotide. Cell number was also unchanged in FCS treated cultures compared to antisense-treated cells.

30 mins:

8 hours:



48 hours:

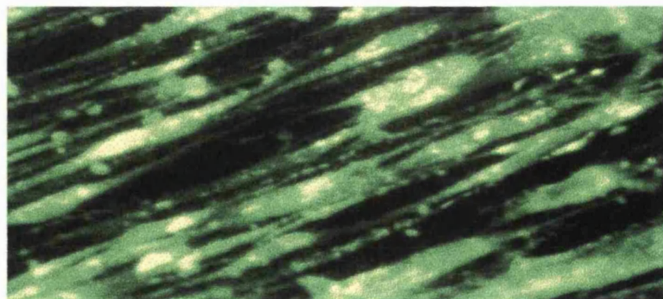


Fig 6.10: Delivery of fluorescently tagged antisense into human dermal fibroblasts using naked DNA:

Photographs showing confocal microscopy of human dermal fibroblasts incubated with 2μm naked FITC-tagged antisense to α2 integrin subunit. Cells were incubated and fixed at varying time points over 72 hours. Antisense was delivered in serum-free media. All micrographs are shown at 640x magnification; 8 sections.

Time following a/s application	*Serum control*	*Stauro control*	*Antisense treated*
0 hours	0.61±0.11	0.60±0.12	0.65±0.09
8 hours	0.62±0.08	0.48±0.15	0.68±0.07
16 hours	0.64±0.06	1.35±0.09*	0.68±0.09
24 hours	0.66±0.10	2.10±0.31*	0.69±0.08
48 hours	0.68±0.09	3.71±0.20*	0.71±0.11
72 hours	0.71±0.09	3.95±0.27*	0.75±0.13

Table 6.1: LDH assay demonstrating that antisense oligonucleotides do not affect cell viability in human dermal fibroblasts.

Table 6.1 shows data derived from a lactate dehydrogenase (LDH) assay to test for cell viability following incubation with antisense oligonucleotides for up to 72 hours. Antisense was added to the cultures at a concentration of 2μM at time 0. Staurosporine (2μM) (stauro) treated cultures were also assayed, as a positive control. Data is represented as arbitrary spectrophotometric units, read at 492nm. Experiments were conducted 3 times for each condition at every time point to gain a mean and SEM. * denotes $p<0.001$ level of significance compared with serum alone control cultures.

6.2.6 Addition of antisense to α2 integrin subunit inhibits expression of total and cell-surface α2 protein

Figures 6.11 and 6.12 show the effects of α2 integrin subunit antisense oligonucelotides on levels of α2 protein in human dermal fibroblasts. The following data shows a time course of Western blots for total α2 integrin subunit over 72 hours. Antisense was incubated with the cultures for 24 hours in serum-free media, prior to the start of the experiment. Media was removed and replaced with 10% FCS. Cells were harvested at 4, 16, 24, 48 and 72 hour time points from the time of removal of antisense (time 0). Western blots indicate that α2 subunit expression was unaffected after 4 hours in the presence of antisense oligonucleotide in all cultures. However, α2 levels were reduced at 16 hours in antisense compared to control cultures, in both the loaded and unloaded samples. By 24 hours, the total α2 levels in antisense-treated cultures were virtually undetectable in loaded and control cultures compared to 10% FCS-treated controls. This significantly reduced expression of total α2 protein was maintained until 72 hours.

Figs 6.12 show western blots of identical experiments to those described above, but assessing cell-surface α2 protein expression only. No change was seen in any cultures in the presence of antisense at the 4 hour time point. At 16 hours in control (untreated) cultures, mechanical load significantly enhanced cell surface α2 protein, as described in the previous chapter. At 16 hours, cell surface α2 was reduced to the same level in loaded and unloaded by antisense treatment. Levels of cell surface α2 in cultures treated with antisense at 24 and 48 hours was virtually undetectable. By 72 hours, expression was becoming evident in antisense treated cultures.

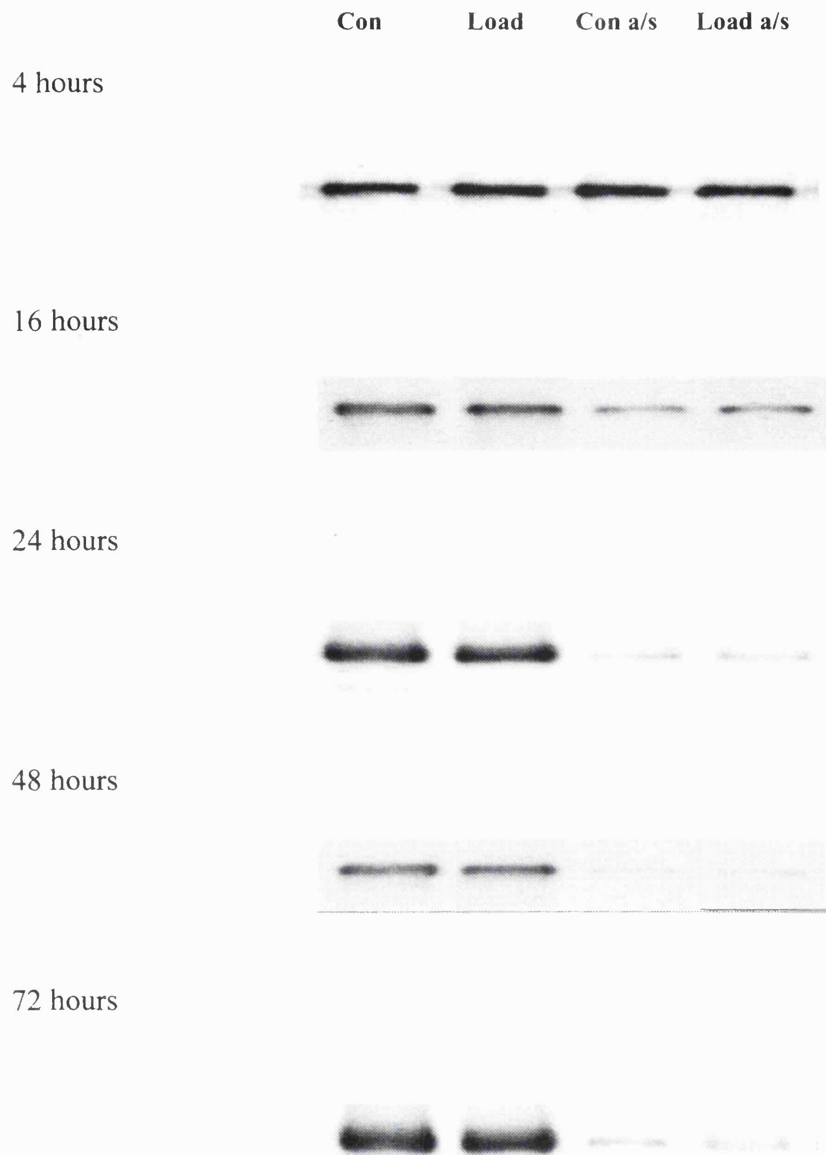


Fig 6.11: Addition of antisense to α2 integrin subunit inhibits expression of total α2 protein

Western blots showing time course of total α2 integrin subunit expression in response to serum, with (load) and without (control) mechanical load and in the presence of antisense (a/s). Cells were incubated with serum-free media containing naked antisense oligonucleotide at 2μm concentration for 24 hours. Antisense was then removed and replaced with serum containing 10% FCS, and cultures were lysed over a time course up to 72 hours. 30μg total protein was used in each well for analysis. Experiments were repeated 3 times for each condition.

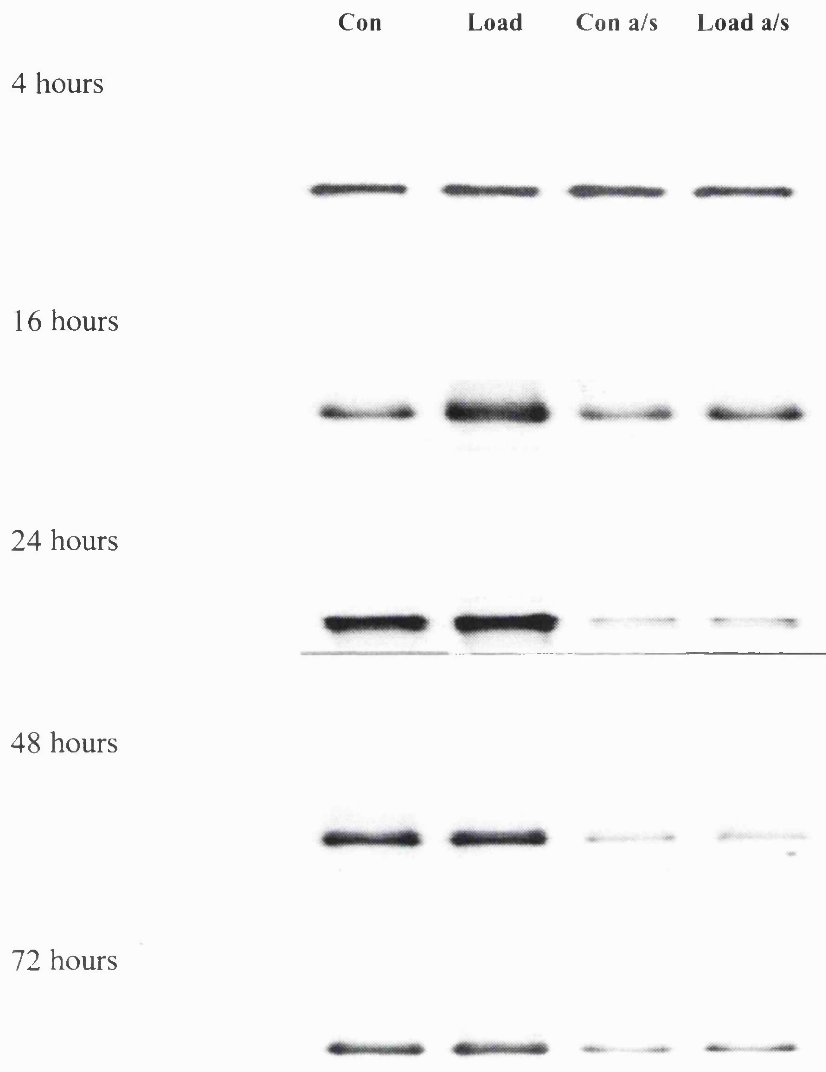


Fig 6.12: Addition of antisense to α2 integrin subunit reduces cell-surface expression of α2 protein.

Western blots showing time course of cell-surface α2 integrin subunit expression in response to serum, with (load) and without (control) mechanical load and in the presence of antisense (a/s). Cells were incubated with serum-free media containing naked antisense oligonucleotide at 2µm concentration for 24 hours. Antisense was then removed and replaced with serum containing 10% FCS, and cultures were lysed over a time course up to 72 hours. 30µg total protein was used in each well for analysis. Experiments were repeated 3 times for each condition.

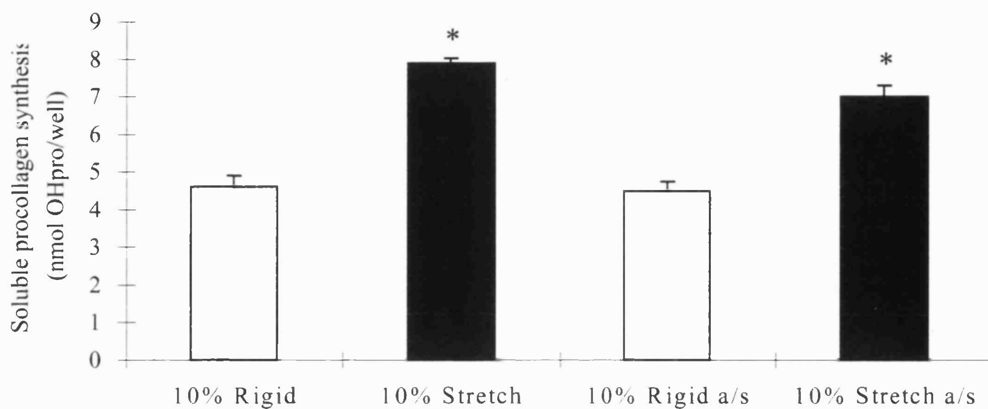
6.2.7 Antisense to α2 integrin reduces collagen synthesis and deposition in loaded samples only

The following data in Fig 6.13 show the effects of antisense to α2 integrin subunit on procollagen production and insoluble collagen deposition.

Fig 6.13 shows the effects of antisense to α2 integrin on soluble procollagen production and insoluble collagen deposition after 48 hours incubation in 10% FCS. Soluble procollagen synthesis (media fraction) was unaffected in control unloaded antisense-treated cultures as compared to serum controls. Mechanically loaded cultures treated with antisense showed a reduction in procollagen synthesis, but not a significant change from loaded serum control levels.

Insoluble collagen deposition (cell layer fraction) was unchanged in unloaded antisense-treated cultures compared to unloaded serum controls. However, the increase in collagen deposition in mechanically loaded cultures was reduced from 89±4% to 31±3% ($p<0.05$) in antisense-treated cells compared to serum controls. Mechanically loaded cultures treated with antisense still displayed a significant increase in insoluble collagen deposition ($p<0.01$) compared to unloaded antisense control after 48 hours.

Soluble procollagen



Insoluble collagen deposition

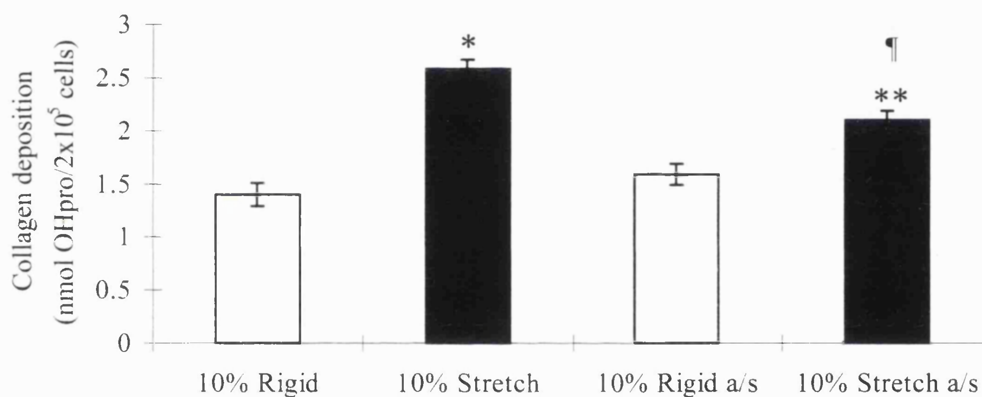


Fig 6.13: Antisense to $\alpha 2$ integrin reduces procollagen production and deposition in loaded samples only

Graphs showing soluble procollagen (media fraction, top panel) and insoluble collagen deposition (cell layer fraction, bottom panel) in cultures treated with antisense to $\alpha 2$ integrin subunit. Cells were incubated with serum-free media containing naked antisense oligonucleotide at 2um concentration for 24 hours. Antisense was then removed and replaced with serum containing 10% FCS. Collagen synthesis was quantitated in all cultures following 48 hours incubation. Data is represented as nmol OHpro/$2x10^5$ cells. There was no change in cell number between control and antisense treated cultures. Experiments were repeated twice. * and ** denote $p<0.001$ and 0.01 respectively compared to control levels. ¶ denotes $p<0.05$ level of significance compared to loaded serum control.

6.2.8 Antisense to α2 integrin inhibits load-induced PCP protein levels

Fig 6.14 shows the effects of antisense oligonucleotide to α2 integrin subunit on PCP and PCPE expression after 24 hours incubation. Western blots indicate that PCP expression is significantly increased in loaded vs. control cultures in the presence of serum. However, on addition of antisense to these cultures, the load-induced increase in PCP protein expression was decreased almost to antisense unloaded control levels. Antisense control PCP levels were unchanged compared to serum control levels. PCPE protein expression was unaffected by the presence of antisense to α2 integrin in both control and loaded cultures.

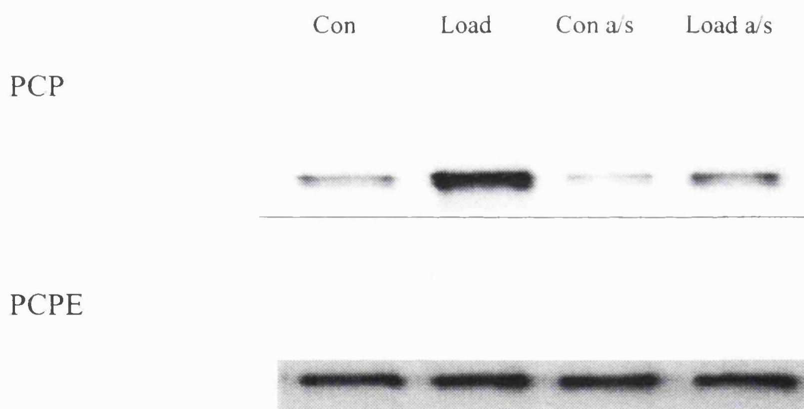


Fig 6.14: Antisense to α2 integrin inhibits load-induced PCP protein levels:

Western blots of PCP and PCPE protein following 24 hours with (load) and without (control), mechanical load in the presence (a/s) or absence of antisense oligonucleotide to α2 integrin subunit. Antisense was incubated with cultures in serum-free media for 24 hours prior to the start of the experiment. This was then replaced with media containing 10% FCS. Cultures were harvested after 24 hours. 30ug of protein was loaded per well, and identical loading was confirmed using ponceaus stain of the final blot. Experiments were repeated 3 times for each condition.

6.3 DISCUSSION

The involvement of integrins in transducing a mechanical stimulus into a biochemical response is currently very poorly understood. There are very few studies that have addressed the role of integrins in stretch-mediated responses such as increased matrix synthesis. However, there have been many studies investigating the role of integrins in normal matrix synthesis, and this information may be vital in the elucidation of mechanosensory mechanisms.

As has been shown in previous chapters, mechanical load increases procollagen synthesis and deposition, as well as procollagen c-proteinase (PCP), the key enzyme involved in insoluble collagen formation. In this part of the study, the aim was to characterise the involvement of specific integrins in the transduction of a mechanical signal into a biochemical one, in the form of increased synthesis and deposition of collagen. The integrins investigated in this context were $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha5\beta1$, as these integrins have been previously shown to be important in the stimulation of stretch-induced signalling cascades using functional blocking antibodies and peptides (MacKenna *et al*, 1998; Wilson *et al*, 1997; Chen *et al*, 1999). These integrins were also chosen in this study as both $\alpha1\beta1$ and $\alpha2\beta1$ are collagen receptors and therefore would logically be involved in procollagen synthesis by fibroblasts plated on a collagen matrix. Conversely, $\alpha5\beta1$ was also investigated in the context of responses to load, as a fibronectin receptor provides an interesting control to test the theory that collagen integrins alone are involved in collagen deposition.

The role of integrins in stretch-induced increases in procollagen synthesis and insoluble collagen deposition was investigated, and data indicated that both $\alpha1\beta1$ and

$\alpha2\beta1$ are involved in collagen synthesis in different capacities, whereas $\alpha5\beta1$ is not. $\alpha1\beta1$ is involved in the stimulation of procollagen synthesis in response to serum and TGFß, as demonstrated by the inhibition of procollagen synthesis in all cultures treated with blocking antibodies to $\alpha1\beta1$. However, procollagen processing was unaffected in these cultures. This would agree with studies which have previously shown $\alpha1\beta1$ to be involved in the downregulation of collagen synthesis collagen gels by both blocking antibodies and $\alpha1$ null cells (Langholz *et al*, 1995). This was confirmed by the observation that $\alpha1\beta1$ blocking antibodies did not affect either PCP or PCPE protein expression either in response to growth factors or mechanical load. This is a very important finding for a number of reasons. It indicates that procollagen and PCP are regulated by different mechanisms, certainly in terms of cell membrane receptor-ECM signalling. It also indicates that $\alpha1\beta1$ is not involved in specific mechanical transduction, but rather in the regulation of procollagen synthesis in fibroblasts.

Interestingly, data indicated that $\alpha2\beta1$ was less involved in the regulation of procollagen synthesis, but involved in the stretch-induced increase in insoluble collagen deposition. Furthermore, the $\alpha2\beta1$ receptor appears to play a key role in the mechanical regulation of PCP expression. Data in this chapter has demonstrated that inhibition of the $\alpha2$ integrin subunit using both antibodies and antisense oligonucleotides successfully inhibits load-induced collagen deposition and load-induced PCP levels without affecting unloaded control levels. This suggests that the $\alpha2$ integrin subunit is directly involved in the detection and transduction of a mechanical signal, and conversion of this signal into an increase in PCP and subsequently collagen deposition.

$\alpha 2$ integrin subunit has been previously reported to be potentially involved in the feedback regulation of procollagen synthesis via binding to the C-terminal propeptide (Weston *et al*, 1994). This has never been conclusively proven to be an inhibitory or stimulatory effect, as the nature of the response is dependent on the specific peptides involved (Weston *et al*, 1997; Bhattachyryya-Pakrasi *et al*, 1998). However, it is certain that this subunit can bind and interact with both the intact procollagen molecule, and indeed terminal cleavage peptides (Weston *et al*, 1997). The data presented here would not agree nor disagree with the concept of propeptde feedback via the $\alpha 2$ integrin as binding to the procollagen molecule was not measured. However, it would appear that blocking the binding sites on this receptor does not alter normal procollagen synthesis or deposition in response to growth factors, which may suggest that in this system, $\alpha 2$ is not playing a role in feedback.

The successful application of antisense oligonucleotide technology in this system also demonstrated that $\alpha 2$ depletion at the protein level did not alter normal collagen deposition, rather it specifically reduced load-induced deposition and PCP expression. Whilst the fundamentals of the use of antisense are still under some debate, data in this chapter clearly demonstrated a reduction in protein levels without any subsequent change in cell viability. Antisense application, whilst completely inhibiting total $\alpha 2$ protein synthesis, did not completely inhibit the expression of this integrin at the cell surface, presumably due to the slow nature of turnover in this receptor. As western blots indicated, a very small amount of $\alpha 2$ subunit was still present at the cell surface after 48 hours antisense treatment. It is possible that this residual integrin expression represented enough receptor mass to stimulate the increased collagen deposition in

loaded cultures. Certainly this would explain why blocking antibodies to $\alpha2\beta1$ integrin totally inhibited load-induced collagen deposition compared to a 70% reduction in antisense-treated cultures. However, the combined data supports a role for $\alpha2\beta1$ in the transduction of a mechanical signal and specific conversion of that signal to an increase in collagen deposition.

The control of PCP expression by integrins has not previously been investigated. The regulation of load-induced PCP expression by $\alpha2$ is therefore novel. PCP has been demonstrated to be upregulated by many of the same factors which regulate procollagen synthesis but has been shown to be temporally regulated differently to procollagen expression (Lee *et al*, 1997). This finding is also reported in this thesis, as PCP expression is enhanced in response to load at 24 hours, whereas procollagen synthesis is enhanced in identical cultures at 48 hours. This suggests PCP and procollagen synthesis may be regulated in a different manner in response to mechanical load, and indeed the data presented in this chapter and chapter 4 confirms this hypothesis.

The exact nature of the regulation of load-induced PCP levels by $\alpha2\beta1$ can only be hypothesised at this time. PCP is known to bind directly to the C-terminal propeptide of procollagen at the cell surface and enhance the rate of cleavage of the peptide by up to 10-fold (Kadler, 1995). It is possible therefore, that in this sequence of events, both procollagen and PCP are able to bind to $\alpha2\beta1$ on the cell surface, which acts as a form of "docking" protein. This may then be responsible for bringing the two molecules closely together, and thereby enhancing the cleavage of the peptide, and consequently increasing the levels of deposited collagen. As mechanical load has been shown to increase both PCP and $\alpha2\beta1$ integrin levels at the cell surface, it is logical that in this

system where these two molecules are forced into close proximity with procollagen, insoluble collagen formation is dramatically enhanced.

Interestingly, α5β1 was not involved in any of the responses to mechanical load, either in terms of procollagen synthesis or deposition. α5β1, a fibronectin receptor, may be more important in the regulation of proliferative or cell cycle/ survival signals in response to load, as have been previously reported in fibroblasts (Wilson *et al*, 1996). The nature of matrix upon which the cells are grown may also influence the specific integrins used in the detection and mediation of forces. Similarly, integrins such as α5β1 may also be involved in the regulation of matrix proteins other than procollagen, such as fibronectin or vitronectin.

The question of how specific integrins are involved in specific cellular responses is very poorly understood. Whilst it is known that different integrins have affinities for different matrix molecules, the reason for multiple integrins binding and signalling from the same substrate is under investigation. α1β1 and α2β1 integrins both bind collagen, and whilst it could be assumed that this represents an example of redundancy, data presented in this thesis demonstrating distinct roles for each receptor would disagree with this assumption. Therefore, specific integrins must stimulate specific responses, despite binding the same substrate.

Recent studies have also demonstrated that specificity of integrins is vital in modulating the activation of growth factors. The epithelially expressed αvβ6 integrin has been shown to bind and activate latent TGFß (Munger *et al,* 1999), as well as binding fibronectin and vitronectin. This regulation was demonstrated in a mouse lacking in αvβ6 integrin, and these animals developed exaggerated inflammation and

were protected from bleomycin-induced pulmonary fibrosis. Integrins $\alpha5\beta1$ and $\alpha v\beta3$ however, also fibronectin receptors, do not activate TGFß, demonstrating specificity of $\alpha v\beta6$ for this growth factor. Similarly, a specific subset of integrins within the same ß1 subunit family are involved in differentially regulating cell cycle progression through G1 phase. For instance, the $\alpha5\beta1$ integrin has been shown to be specifically involved in counteracting the apoptotic effects of serum starvation in cells plated on a fibronectin matrix (Zhang *et al,* 1995). $\alpha5\beta1$ acts via FAK phosphorylation to elevate levels of the survival factor Bcl-2, as compared with $\alpha v\beta1$, $\alpha2\beta1$ and $\alpha1\beta1$ which do not stimulate expression of this survival factor, and therefore permit cell cycle arrest and apoptosis, even in the presence of mitogens (Wary *et al*, 1996). These examples highlight the wide range of different actions and the specificity of integrin receptors.

This specificity is also true of both $\alpha1\beta1$ and $\alpha2\beta1$ integrins. Both integrins bind RGD sequences in collagen (Gullberg *et al*, 1992) and stimulate FAK phosphorylation (Schaller *et al*, 1992). However, $\alpha1\beta1$ is distinct from $\alpha2\beta1$ only in being able to recruit the adapter protein Shc and activate MAP kinase, independently of FAK, via the Grb2-Sos-Ras pathway (Pozzi *et al*, 1998). As MAP kinases and AP-1 transcription factors have been implicated in the regulation of collagen genes (Davis *et al*, 1996; Chung *et al*, 1996) it is possible that this represents the molecular mechanism of $\alpha1\beta1$ regulation of basal collagen synthesis. This would also suggest that load-induced regulation of collagen deposition is Shc independent, as it is regulated via $\alpha2\beta1$. Less is known about the specific molecules involved in signalling following $\alpha2\beta1$ activation. The elucidation of the signalling proteins specific to $\alpha2\beta1$ would assist in the understanding of the mechanical stimulation of PCP activation.

In conclusion, the data presented here has shown a number of very interesting observations. In this system, where dermal fibroblasts are plated on collagen, $\alpha1\beta1$ is the receptor responsible for regulating procollagen synthesis in unloaded and loaded cells alike. However, $\alpha2\beta1$ is specifically involved in the regulation of collagen deposition in response to load, and has no role in the control of these processes in unloaded cells. These data confirm the hypothesis stated at the beginning of this thesis that integrins are involved in the deposition of collagen by cells under mechanical tension. The role of the integrin $\alpha2\beta1$ in the regulation of collagen deposition and fibrotic processes *in vivo* has yet to be investigated. However, it is clear both from data present in this thesis and previously published work that integrins play a crucial role in the transduction of mechanical signals. The exact nature of this relationship in *in vivo* pathological situations must be investigated to gain further understanding of these processes.

CHAPTER SEVEN

Results

Collagen synthesis and alignment by 3D load

7.1 INTRODUCTION

Clinical studies have demonstrated that a reduction in the mechanical tension surrounding a wound area can minimise mature scar size (Meyer and McGrouther, 1991). It has also been established that the orientation and physical positioning of a wound *in vivo* dramatically influences the shape of the wound following closure. This has been attributed to the different mechanical tensions apparent within different parts of the body, which exert strains on the wound environment. (Sommerlad and Creasy, 1978. Kengesu *et al*, 1993). These studies have shown histologically that collagen fibres within scar tissue are aligned in comparison with surrounding dermis. This suggests that external forces are playing a part in the final orientation of the matrix in scar tissue.

As described in chapters 3-6, mechanical load *in vitro* has been shown to activate cells derived from tendons, ligaments and the lung (Bishop *et al*, 1993, Gilbert *et al*, 1994), increase total procollagen synthesis in cardiovascular fibroblasts (Butt and Bishop, 1997). As well as this, load stimulates replication and growth factor production in smooth muscle and endothelial cells (Wilson *et al*, 1993; Sumpio *et al*, 1987). Chapters 3-6 demonstrated that 2D load stimulates collagen deposition in dermal fibroblasts via regulation of integrins. It is hypothesised that load controls the final fibroblast and collagen orientation as well as the collagen production rate. However, this hypothesis has never been tested in an *in vitro* system prior to this thesis.

Fibroblasts grown in free-floating/non-tethered retracted collagen lattices, a non-tensioned *in vitro* model of wound contraction, demonstrate a decrease in collagen

synthesis at both transcriptional and translational levels, a decreased collagenase activity and changes in cell morphology (Eastwood *et al*, 1996; Mauch *et al*, 1988; Nakagawa *et al,* 1989). The changes in matrix and MMP production have been attributed to a "mechanotransduction" effect via specific integrins. Tethered collagen lattices are also used in some studies as a model of tension in comparison with the tensionless contracted lattice model (Lambert *et al*, 1992). However, the tethered model represents non-quantifiable endogenous cell-generated tension only, and does not apply exogenous load to fibroblasts.

There is a wealth of published data on the effect of contraction of collagen gels on fibroblast response to growth factors. Many modifications of the collagen lattice have been used in order to attempt to bring it closer to an *in vitro* dermal equivalent model for analysis of wound healing and tissue repair. For example, the addition of other matrices into the lattice, such as fibronectin and fibrin, as well as the addition of exogenous growth factors normally found at sites of injury such as PDGF (Tuan *et al*, 1996; Clarke *et al*, 1989; Montesano *et al*, 1988). However, none of these studies have addressed the question of how defined exogenous mechanical loading may affect fibroblast matrix synthesis and proliferation. Contraction and exogenous loading both occur during tissue repair and previous studies have shown that *in vitro* these two physical influences can often exert very different effects on cells. In order to address this, we have taken a system which encompasses both the contractile properties of *in vivo* granulation tissue (Bell *et al*, 1979), and the exogenous loading which occurs during muscle and dermal movement (Eastwood *et al*, 1996).

It has previously been shown that with the use of a computer-controlled tensional-loading device capable of applying small unidirectional loads across fibroblast seeded

collagen gels it is possible to predict cell alignment *in vitro*. In this system, cells adopt a bipolar alignment parallel with the steepest contours of the iso-strain generated in the gel (Eastwood *et al*, 1998). However, although cellular morphology has been investigated in this system, the responses to load in terms of new matrix synthesis and organisation have not. Elucidation of the mechanisms, and mediators, of the effects of load may help to clarify some pathological mechanisms and indicate novel therapies for abnormal collagen deposition and scar formation.

Aims

This study aims to examine the direct effect of mechanical forces in combination with serum and TGFß1 on procollagen synthesis in conjunction with alignment of collagen fibres and dermal fibroblasts cultured within a 3-dimensional collagen matrix. This study also aims to investigate the relationship between load-induced collagen synthesis and fibre alignment by chemically inhibiting or reducing new collagen synthesis and assessing subsequent collagen alignment following load.

7.2 RESULTS

7.2.1 Fibroblasts embedded in collagen gels had an increased rate of cell proliferation.

Collagen gels were set up as described in Materials and Methods in Chapter 2. Contracted control gels were allowed to freely contract over the 48 hours incubation period. Mechanically loaded gels were subjected to the cyclical overload programme (see Fig. 2.7) for up to 48 hours. Fibroblasts were then isolated from each gel for analysis. All collagen gels were incubated with 10% FCS for the entirely of each experiment.

Fig 7.1 demonstrates that there was an increased number of cells in the first 24 hours in both contracted control and loaded gels. Following this, there was no significant increase in cell number after 24 hours culture in contracted collagen gels. The cell proliferation in contracted gels over 48 hours showed an overall 47±7% increase over the 48 hour culture period. However, the mechanically loaded cell number increased by 70±9% over the 48hour period. Therefore, by the 48 hour time point, cell number within the mechanically loaded gels had increased by 24±4% ($p<0.01$) compared to unloaded control gels.

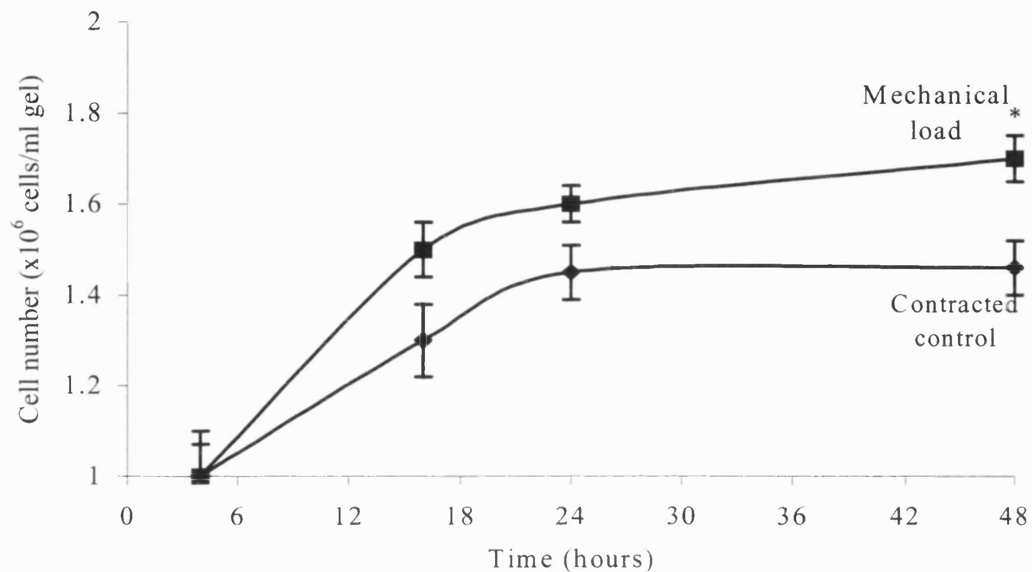


Fig. 7.1: Fibroblasts embedded in collagen gels had an increased rate of proliferation.

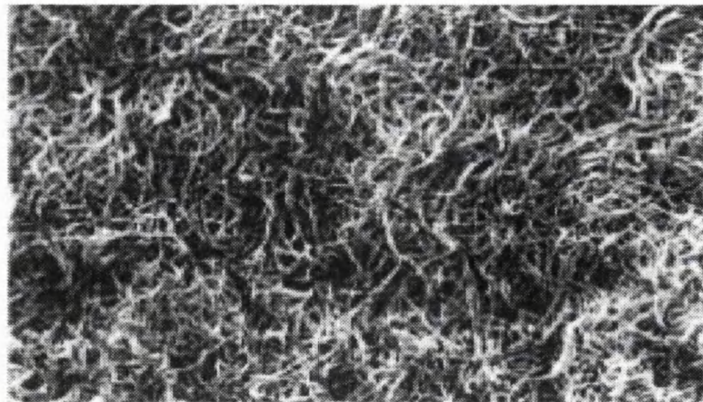
Human dermal fibroblasts were embedded in collagen gels in the presence of serum and allowed to contract or subjected to exogenous mechanical load using the t-CFM. Cell proliferation was measured by conducting cell counts on gels for up to 48 hours. Data is expressed as 10^6 cells/ml collagenover 48 hours. **denotes $p<0.01$ level of significance of mechanical load compared with control (contracted) gel at that time point.

7.2.2 Collagen fibres in 3D gels align in response to mechanical load

Fig. 7.2a shows an electron micrograph of collagen fibres within a contracted collagen gel (endogenous load only) containing human dermal fibroblasts. The micrograph indicates a "basketweave" structure of collagen fibrils after 24 hours, with no definable orientation. Control (cell-free) collagen gels had no collagen fibril alignment after 24 hours loading with or without external loading. Free-floating control gels (minimal endogenous loading) also had no fibril alignment.

Fig 7.2b is an electron micrograph showing alignment of collagen fibrils in response to 24 hours of mechanical loading. Collagen fibrils were aligned following uniaxial cyclical loading after 24 hours in cell-seeded mechanically loaded gels. Alignment was found to be parallel to the direction of the mechanical loading. In addition to this, the alignment of fibrils was found in the area of the gel immediately around cells (though not in the intercellular matrix). All experiments were performed in the presence of 10% FCS. Collagen alignment was assessed under each condition from 4 hours onwards, and occurred after 24 hours of loading.

a]



b]

Direction of mechanical load

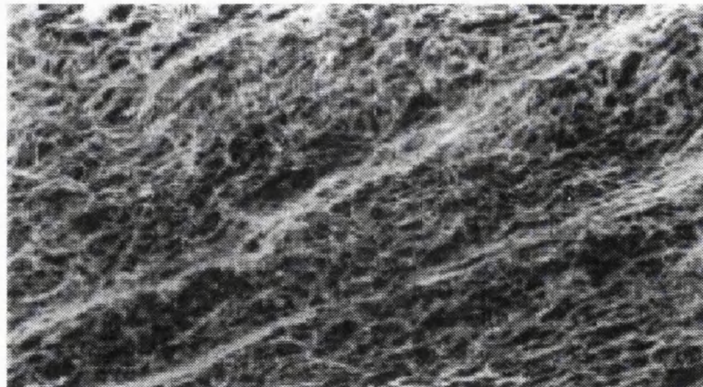


Fig 7.2: Collagen fibres in 3D gels align in response to mechanical load:

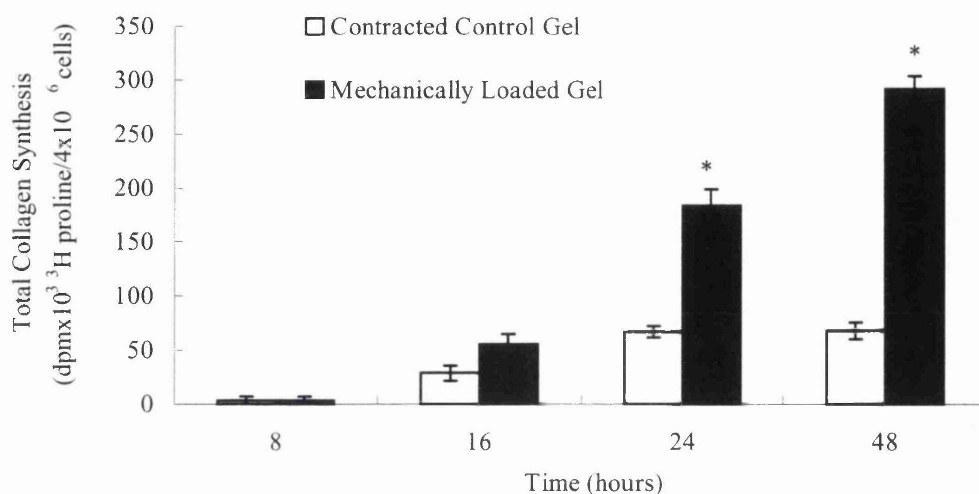
Figs. 7.2a and 7.2b show electron micrographs of collagen gels containing human dermal fibroblasts. The gel in 7.2a has contracted over a period of 24 hours, whereas the gel in 7.2b has been mechanically loaded using the t-CFM for 24 hours. All experiments were performed in the presence of 10% FCS. Control (cell-free) collagen gels had no collagen fibril alignment after 24 hours loading. Three gels were analysed by SEM for each condition.

7.2.3 Mechanical load in the presence of serum stimulates total procollagen synthesis by human dermal fibroblasts in collagen gels

Figure 7.3 shows procollagen synthesis in response to 10% serum in mechanically loaded or contracted collagen gels. The time course data, shown in the top panel, indicated a total collagen synthesis increase relative to unloaded control after 16 hours. This enhancement of collagen synthesis continued to 170±22% and 320±26% after 24 and 48 hours respectively ($p<0.001$). Fibroblasts in loaded collagen gels continued to produce collagen after 48 hours incubation, in contrast to the free-floating control gel in which synthesis virtually stopped during the second 24 hours of incubation.

The rate of total procollagen synthesis is shown in the bottom panel of Fig. 7.3. In the first 24 hours of mechanical load, the rate of procollagen synthesis was 270±40% ($p<0.001$) greater than control free-floating gels and 420±52% ($p<0.001$) greater in the 24-48 hour period. In contracted control gels, the rate of procollagen synthesis was dramatically decreased over the 24-48 hour period.

7.3a



7.3b

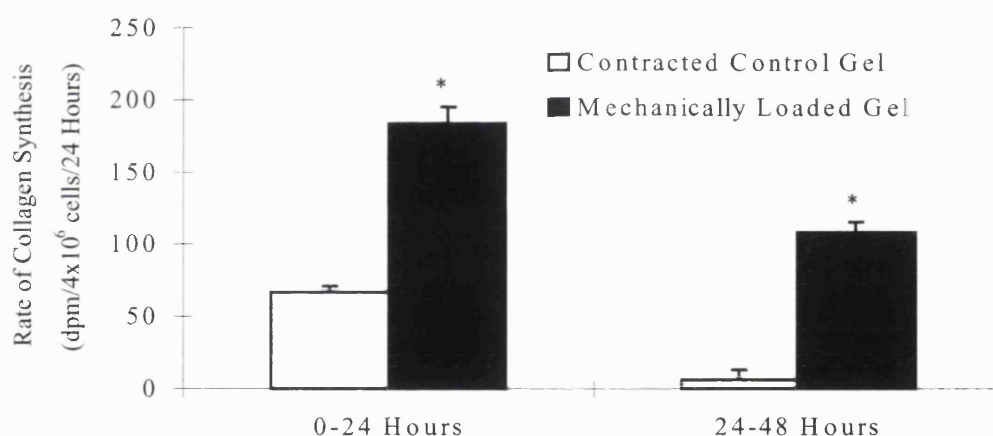


Fig. 7.3: Mechanical load in the presence of serum stimulated collagen synthesis by human dermal fibroblasts in collagen gels:

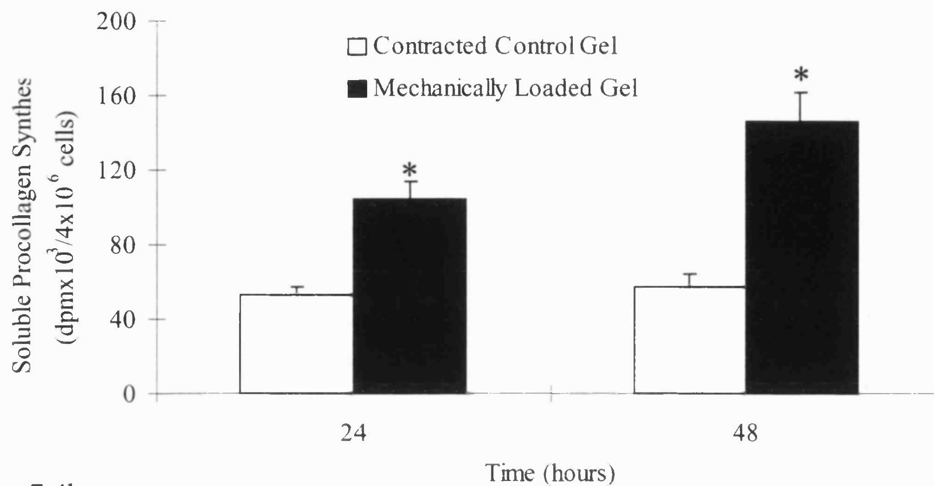
Figure 7.3a shows a time course of total procollagen synthesis in human dermal fibroblasts in collagen gels subjected to contraction or mechanical loading on the t-CFM. Procollagen synthesis was measured by quantitating levels of ^{3}H proline incorporated into hydroxyproline. All data is expressed as ^{3}H hydroxyproline/4x10^{6} cells. Figure 7.3b shows the rate of procollagen synthesis by human dermal fibroblasts in collagen gels subjected to contraction or mechanical load over 48 hours. Data is expressed as ^{3}H hydroxyproline/4x10^{6} cells/24 hours. * denotes $p<0.001$. Open bars denote control gels and solid bars mechanically loaded gels. N=3 for each condition tested.

7.2.4 Mechanical load in the presence of serum stimulates insoluble collagen deposition by dermal fibroblasts in collagen gels

The effect of mechanical load on procollagen processing and partitioning was assessed in fibroblasts in collagen gels over 48 hours. Fig 7.4a indicates soluble procollagen synthesis (media fraction only) was increased in the first 24 hours in loaded gels compared to contract gels by 100±18% ($p<0.001$). In the 24-48 hour period, soluble procollagen synthesis in loaded gels compared to control was increased to 160±24% ($P<0.001$). Fibroblasts in contracted collagen gels did not demonstrate any significant increase in soluble procollagen synthesis between the 24-48 hour period.

Fig. 7.4b shows that insoluble collagen deposition (gel fraction) was enhanced in mechanically loaded collagen gel cultures compared to control gels. There an insignificant amount of insoluble collagen deposited in contracted control gels over the 24-48 hour incubation period. However, deposition into mechanically loaded cultures continued over the 24-48 hours period. Collagen deposition in loaded gels was 305±85% ($p<0.001$) above that found in free-floating gels after the first 24 hours of incubation and approximately 610±82% ($p<0.001$) greater in the 24-48 hour period.

7.4a:



7.4b:

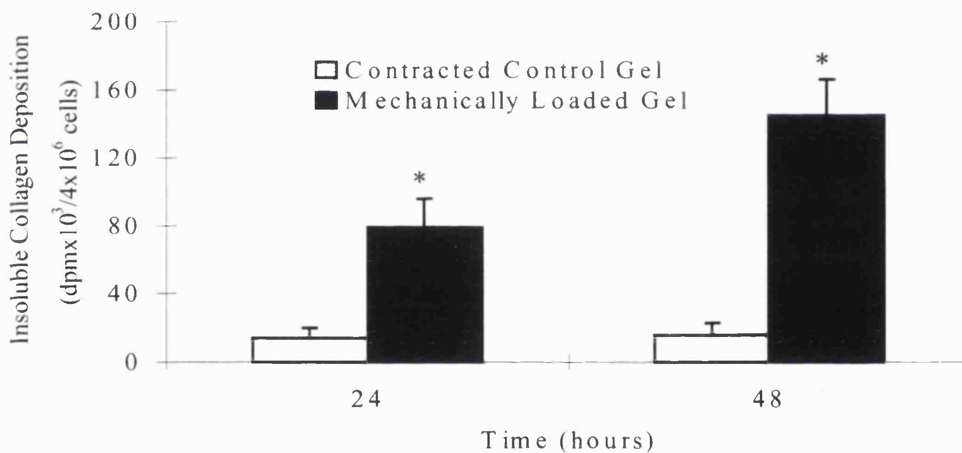


Fig. 7.4: Mechanical load in the presence of serum stimulates insoluble collagen deposition by human dermal fibroblasts in collagen gels:

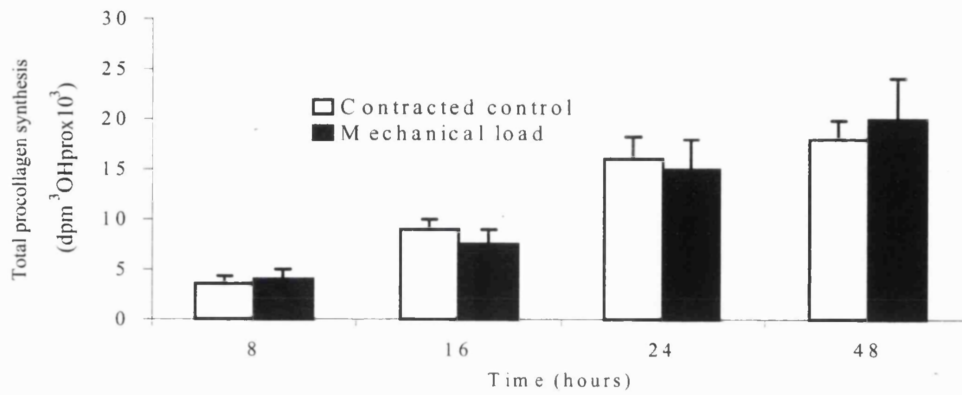
The top panel shows measurement of new soluble procollagen synthesis by cells in collagen gels undergoing contraction or loading. Soluble procollagen was measured as the soluble fraction of ^{3}H hydroxyproline in the media following treatment. Bottom panel shows quantitation of deposition of new insoluble collagen into gels subjected either to contractile forces or exogenous mechanical load. Insoluble collagen was measured as ^{3}H hydroxyproline extracted from the gel following treatment. Data is presented as collagen (nmol ^{3}H OHpro/4x10^6 cells/24 hours). * and ** denotes $p<0.001$ and $p<0.01$ level of significance in mechanically loaded compared to contracted controls respectively. Open bars denote control gels and solid bars mechanically loaded gels. N=3 for each condition tested.

7.2.5 TGFβ1 acts in synergy with mechanical load to enhance procollagen synthesis and fibril alignment

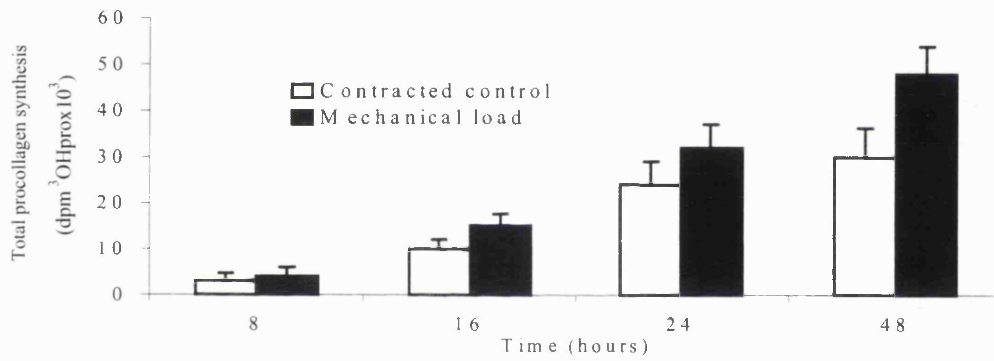
Fig 7.5 shows data demonstrating that TGFß1 at any of the concentrations tested did not significantly stimulate total collagen synthesis in free-floating collagen gels over 16 hours. In cultures mechanically loaded for 24 hours, however, TGFß stimulated collagen synthesis above contracted control levels at 100 and 200pM, by 190±33% ($p<0.001$) and 120±48% ($p<0.01$) respectively. After 48 hours, the fibroblasts mechanically loaded in 100 and 200pM TGFß displayed a further increase in collagen synthesis by 280±85% ($P<0.001$) and 195±65% ($p<0.01$) compared to controls. Lower concentrations of TGFß in the presence of mechanical load did not stimulate collagen synthesis over control levels at any time point.

Figure 7.6 shows an electron micrograph of a gel loaded in the presence of 50pM TGFß1. As the micrograph indicates, no trace of alignment in the gel could be detected. At concentrations lower than 100pM, the mechanically loaded gels showed no identifiable traces of alignment or collagen synthesis. Figure 7.6 indicates in gels treated with 100pM TGFß, the collagen fibrils were aligned parallel with the direction of loading. Alignment was also seen in gels loaded in the presence of 200pM TGFß. Serum-free controls were not used in any of the experiments as growth factors must be present in low concentrations for contraction of the gel to occur. Instead, low concentrations of TGFß1 were used, which still allow contraction, but do not promote new collagen synthesis as shown in Fig. 7.5.

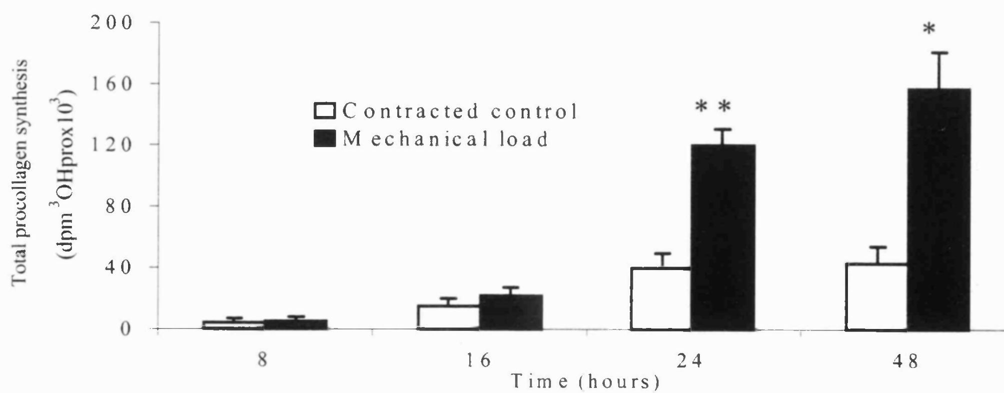
10pM TGFß



50pM TGFß



100pM TGFß



200pM TGFß

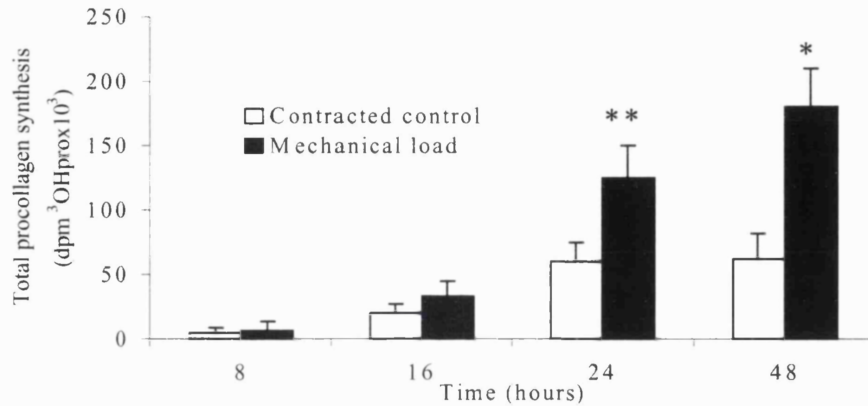
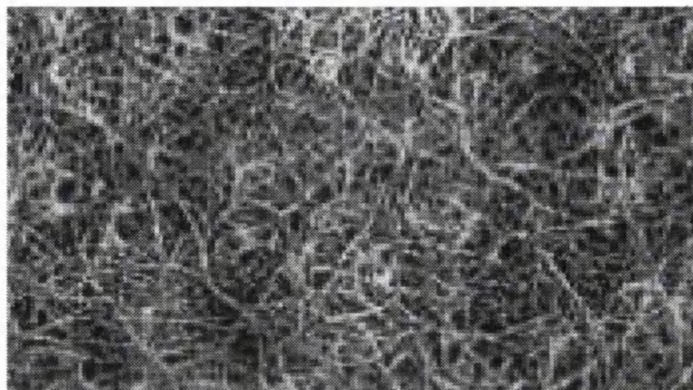


Fig. 7.5: TGFβ acts in synergy with mechanical load to enhance procollagen synthesis:

Figure 7.5 shows the effect of increasing concentrations (10, 50, 100 & 200pM) of TGFß1 in serum-free media on total collagen synthesis. TGFß1 concentrations below 10pM were also investigated (1 and 5pM), but no significant increases in collagen synthesis or alignment were detected (data not shown). All gels contained HDF at a concentration of 10^6 cells/ml collagen. All values are corrected for changes in cell number between experiments. Open bars denote control gels and shaded bars mechanically loaded gels. * and ** denote $p<0.001$ and $p<0.01$ level of significance respectively as compared to contracted control.

50pM TGFβ:



100pM TGFβ:

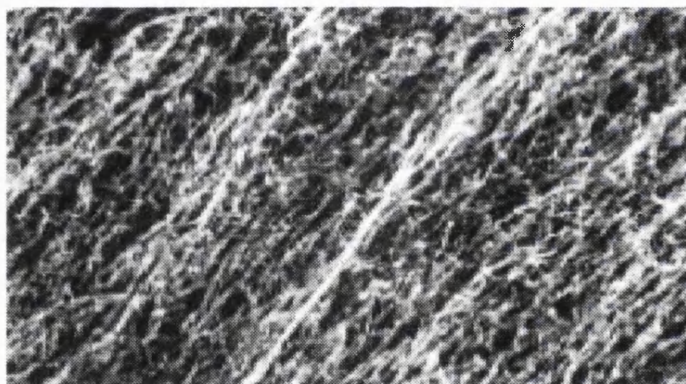


Fig. 7.6: Low concentrations of TGFβ do not support load-induced fibril alignment

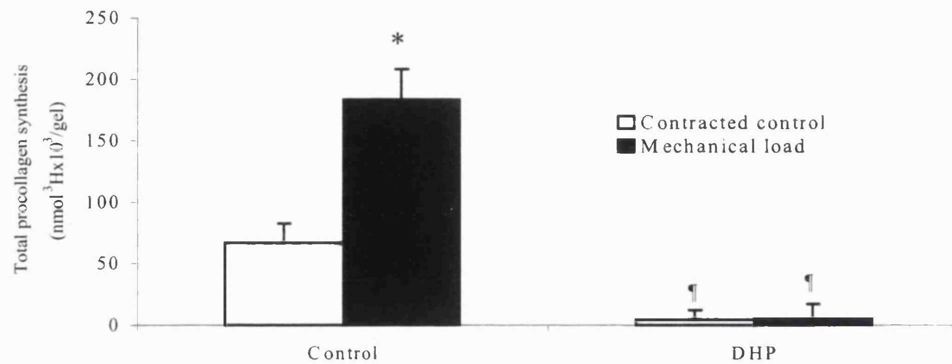
Electron micrographs showing collagen gels containing dermal fibroblasts mechanically loaded for 24 hours in the presence of 50pM (top panel) and 100pM (bottom panel) TGFβ, in serum-free media. Scanning electron microscopy was performed on the fixed gels, and all micrographs are shown at the same magnification (x 20,000).

7.2.6 Chemical inhibition of collagen synthesis by dermal fibroblasts in mechanically loaded collagen gels

Figure 7.7 shows the effects of two procollagen synthesis inhibitors on new collagen synthesis following 24 hours of incubation with each compound. Cells were loaded or contracted within collagen gels in the presence of 10% FCS and one of the two collagen synthesis inhibitors. Inhibition of synthesis was performed using two inhibitors of collagen synthesis (which act at different sites on the procollagen molecule), 3,4 dihydroxybenzoate (EDHB) and 3,4 dehydro-DL-proline (DHP) at 400uM and 1mM respectively. EDHB is a prolyl hydroxylase inhibitor, preventing conversion of proline residues into hydroxyproline, and therefore preventing mature procollagen formation. DHP is a proline analogue, which becomes incorporated into newly formed procollagen in place of proline residues. However, as the proline is conformationally altered, hydroxylation of the residue cannot take place and therefore the procollagen molecule is degraded intracellularly. Inhibitors were added 30 minutes prior to the onset of loading/contraction.

Data indicates that both EDHB and DHP (Figs 7.7a and b respectively) successfully inhibited new procollagen synthesis down to an insignificant level in both mechanically loaded and contracted control gels after 24 hours incubation, as compared to 10% FCS control gels. Inhibitors were not toxic to the cells, as demonstrated using cell counts and lactate dehydrogenase (LDH) cell viability assays.

a] DHP



b] EDHB

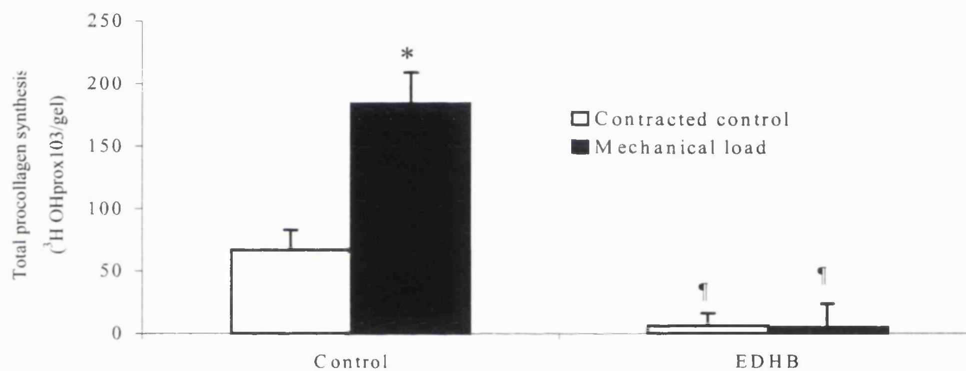


Fig 7.7: Chemical inhibition of collagen synthesis by dermal fibroblasts in mechanically loaded collagen gels:

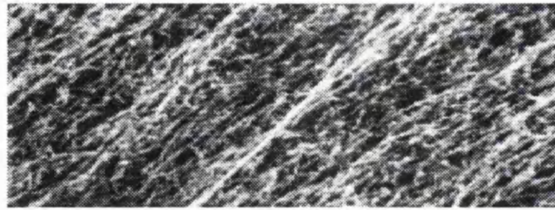
Figures 7.7a and b show collagen synthesis within gels that have been loaded for 24 hours in the presence of two collagen synthesis inhibitors: 3,4dihydroxybenzoate (EDHB) and 3,4 dehydro-DL-proline (DHP) used at 400uM and 1mM respectively. Three gels were analysed for each of the inhibitors tested, and shown to successfully inhibit collagen synthesis by cells within the gels. Procollagen synthesis inhibitors were not cytotoxic as indicated by no change in cell viability or cell morphological changes in the presence of either compound. Data is shown as ^{3}H OHpro levels/gel ($4x10^6$ cells). * denotes $p<0.001$ compared to contracted control levels. ¶ denotes $p<0.001$ level of significance compared to respective non-inhibitor treated control gels.

7.2.7 Inhibition of collagen synthesis inhibits load-induced collagen fibril alignment

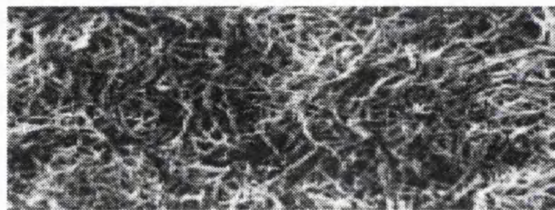
Figures 7.8a-c indicate that inhibitors of new collagen synthesis in dermal fibroblasts can inhibit the alignment of collagen fibrils seen in serum-treated gels in response to load. The inhibition of procollagen with the two compounds, 3,4 dihydroxybenzoate (EDHB) and 3,4 dehydro-DL-proline (DHP), was confirmed using samples of the same gels (data shown previously in Fig 7.7). Figure A is a control gel, mechanically loaded in the presence of 10% FCS only, for 24 hours. The micrograph indicated the aligned collagen fibrils parallel to the direction of the mechanical force as demonstrated previously in Fig. 7.2. Figures B and C are identical cultures, but with EDHB and DHP inhibitors added at 400uM and 1mM respectively.

Electron micrographs indicated that the alignment seen in the control gel in the presence of mechanical load is no longer evident in either gel treated with collagen synthesis inhibitors. Studies on parallel cultures demonstrated no change in cell viability in the presence of either compounds and no changes in cell morphology were seen with each inhibitor.

a] Control



b] EDHB:



c] DHP:

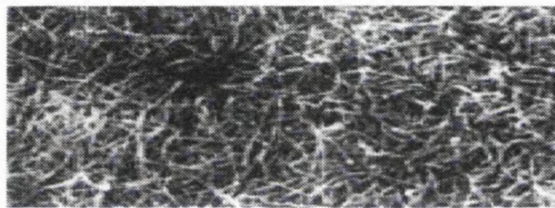


Fig. 7.8: Inhibition of collagen synthesis inhibited load-induced collagen fibril alignment:

Figure 7.8a, b and c are electron micrographs of collagen gels containing human dermal fibroblasts, all subjected to 24 hours mechanical load. Figure 7.8a is a control gel, loaded in the presence of serum only. Figs. 7.8b and c show gels that have been loaded in the presence of two collagen synthesis inhibitors: 3,4dihydroxybenzoate (EDHB) and 3,4 dehydro-DL-proline (DHP) respectively. Procollagen synthesis inhibitors were not cytotoxic as indicated by no change in cell viability or cell morphological changes in the presence of either compound. Experiments were repeated three times for each of the inhibitors.

7.3 DISCUSSION

These data demonstrate that mechanical loading of human dermal fibroblasts embedded in collagen gels can both stimulate collagen synthesis/deposition and collagen fibril alignment. This is in contrast to the response of free-floating collagen gels, where a profound downregulation of procollagen synthesis has been reported (Geesin *et al*, 1993; Eckes *et al*, 1993; Mauch *et al*, 1988 and confirmed in this study).

Scanning electron microscopy indicated that collagen fibril alignment to the axis of mechanical loading only occurred when cells were present. Non-loaded (free-floating) gels and cyclically loaded gels containing no cells exhibited no alignment. These studies suggest therefore that the rearrangement of the type I collagen fibril lattice is a result of the cellular response to load. Dependence of cell and peri-cellular fibril alignment on direction of loading was previously demonstrated within this system (Eastwood *et al*, 1998). However, this is the first demonstration of collagen fibril alignment. There are two possible mechanisms for this system.

Firstly, cells are stimulated to produce matrix proteases in response to load, which begins the remodelling of the matrix. Alternatively, the response may be mechanical whereby directional loading causes cell alignment, and consequently through cell-matrix contacts leads to collagen fibre orientation. A combined mechanism appears most plausible since the biochemical mechanism alone could not supply spatial cues for orientation: mechanics may be responsible for initiating cell alignment and supplying spatial cues, and cells can then achieve collagen fibril alignment (and fixation of this structure) by a biochemical mechanism.

Fibroblasts within the loaded gels are aligned by at least 16 hours in the t-CFM (Eastwood *et al*, 1998). Only after this cell alignment was the alignment of the collagen fibrils seen in the immediate environment of these cells. In addition mechanical loading alone (without cells present) at the stress applied did not produce detectable fibril alignment at all. This suggests the cells align the collagen rather than vice versa. The implications of this are that the cells use matrix in the detection of load, and then translate this into a change in extracellular matrix structure and density. Previous studies, however, have demonstrated that cells can experience contact guidance from the aligned surrounding collagen fibrils within collagen gel systems (Guido and Tranquillo, 1993).

In these studies, it is hypothesised that the mechanical load, which promotes fibril alignment, would then orient cells by contact guidance. Cell contact guidance by aligned collagen fibrils has been shown by Tranquillo and colleagues who use a model of magnetically oriented collagen gels to investigate the directional migration of fibroblasts (Dickinson *et al*, 1994). Recent studies have shown that increasing collagen fibril orientation and cell orientation parallel to the collagen fibrils is associated with a decrease in cell migration through the lattice (Barocas and Tranquillo, 1997). Contact guidance is likely to be occurring in the culture force monitor reported here. However, it would seem that cell alignment is dictating collagen alignment in our system, and therefore may involve an earlier event, which governs this process. Contact guidance may indeed be important for governing cell alignment during tissue repair or engineering (Brown *et al*, 1997). Likewise, understanding the mechanisms involved in the actions and reactions of fibroblasts when placed under mechanical load are also vital in the development of these processes.

The effects of load on the synthesis and deposition of collagen within this system was also determined. HPLC fractionation to separate and quantitate newly synthesised collagen in the samples produced data showing a 2.3-fold increase in total collagen synthesis in loaded versus control gels. This analysis also established that the loading altered the partitioning of soluble and insoluble collagen. Loaded gels contained almost twice as much precipitable new collagen as unloaded controls over 24 hours. This indicated an effect of load on the processing of soluble into insoluble collagen. As demonstrated in chpaters 3-6, this phenomenon occurs in the 2-dimensional loading system. An increase in procollagen c-proteinase (PCP) levels and activity led to an increase in insoluble collagen deposition by dermal fibroblasts in this system. It seems likely that a similar mechanism could operate in this 3-dimensional system to produce elevated procollagen conversion.

The interdependancy of induced fibril orientation and new collagen synthesis was first suggested by the TGFß experiments and then tested by blocking collagen synthesis using either a proline analogue or prolyl hydroxylase inhibitor added to the loaded collagen gel. Proline analogues prevent hydroxylation of proline residues on newly synthesised procollagen thereby preventing normal triple helix formation. Underhydroxylated collagen is rapidly degraded within the cell (Berg et al, 1980). Specificity of hydroxyproline to the procollagen molecule means that non-collagen protein synthesis is unaffected. Prolyl hydroxylase inhibitors prevent the hydroxylation of the proline residues, thereby again rendering the new procollagen molecule biologically ineffective and susceptible to intracellular degradation. These collagen synthesis inhibitors have been used independently but to the same effect in other systems (Sasaki et al, 1987; Nandan et al, 1990; Franceschi et al, 1994).

In this chapter, data indicates that both of the compounds successfully inhibited collagen synthesis and in the present system also inhibited load-induced collagen fibril alignment. This demonstrates that synthesis of new collagen is necessary for the cells to begin to reorganise and remodel the existing collagenous network. This finding has considerable implications for the mechanisms by which fibroblasts re-organise existing collagen networks, a process which is currently very poorly understood.

Data presented in this chapter also demonstrates that TGFß1 stimulates fibroblast procollagen synthesis, and this is enhanced under uniaxial loading. Numerous reports have shown enhanced collagen production by TGFß and some have identified increased fibroblast contraction rate of collagen gels (Montesano and Orci, 1988). In these studies, however, TGFß attenuates fibroblast collagen synthesis, as well as increasing cell-surface levels of integrin α2β1, which may then be responsible for increased contraction (Riikonen *et al*, 1995). Data presented in this thesis indicates that if the cells are exogenously mechanically loaded whilst exposed to TGFß the downregulation of collagen production can be reversed. This implies that the cells are somehow activated in response to load, enhancing their response to TGFß and increasing procollagen production. This TGFß-mediated "activation" may involve changes in integrin expression and focal adhesion formation in response to load, which in turn alters cellular interactions with the surrounding extracellular matrix.

The involvement of α1β1 and α2β1 integrins in the responses to 3D load that is reported in this chapter is currently unknown. Most studies in the past have

concentrated on α1β1 and α2β1 as the primary transducers of surrounding mechanical tension in collagen lattice systems. Recently, it has been shown that α1β1 regulates the collagen downregulation during contraction, whereas the actual contraction process itself is mainly governed by α2β1 (Klein *et al*, 1991; Langholtz *et al*, 1995). Based on evidence in Chapters 5 and 6 of this thesis, and these publications, it could be hypothesised that α2β1 is involved in both the mechanical contraction of collagen lattices, and indeed the detection and transduction of a mechanical signal. Clearly it is highly feasible that α2β1 may be responsible for controlling mechanically induced collagen deposition by dermal fibroblasts within *in vitro* systems. As data has demonstrated in this chapter, stretch-induced collagen deposition is necessary for the corresponding collagen fibril alignment to occur in this system. Further investigations must be conducted to ascertain the involvement of the α2β1 receptor in mechanotransduction in a 3D system, and the potential regulation of collagen deposition and alignment in response to mechanical forces in dermal repair *in vivo*.

The present study shows that the presence of cells and their ability to produce collagen in response to load are directly related to the alignment of collagen fibrils. It is concluded that new collagen synthesis and load-dependent fibril alignment are closely interrelated. This could be extrapolated to suggest that fibroblasts under exogenous mechanical load *in vivo* will deposit increased collagen, enabling collagen fibrils to become aligned parallel to the direction of the force. This has implications in the context of wound healing and dermal scar formation, where collagen organisation is known to be altered (Sommerlad & Creasey, 1978). These effects have important implications for understanding the role of mechanical loading and the biological consequences of stretch in scarring.

CHAPTER EIGHT

General discussion

8.1 DISCUSSION

The healing of wounds in most terrestrial vertebrates occurs by scar formation. The scar serves as a patch rather than a replacement for restoration of structural integrity to the tissue. Scars can be problematic if they are too weak, too strong (thereby compromising other structures) or too abundant. Understanding the biology of dermal tissue repair would have great value in the design of potential therapies to combat the pathological and cosmetic problems associated with scar formation. Current insights into the mechanisms governing the final deposition of collagen into an ordered scar are poorly understood. It has been recognised for many years clinically that mechanical tension surrounding a healing dermal wound can influence the morphology of the final scar. However, this observation has never been tested biochemically in either an *in vitro* or *in vivo* system.

The findings of this thesis demonstrate that two different *in vitro* mechanical loading systems stimulate an increase in procollagen synthesis, deposition and alignment in human dermal fibroblasts. These findings have a number of very important implications both in understanding mechanisms of fibroblast activation *in vitro*, and scarring and fibrotic disorders.

8.1.1 The in vitro *regulation of collagen deposition by load:*

The data presented in this thesis has demonstrated the powerful effects that mechanical load can exert upon fibroblast function. In both two and three-dimensional

loading systems, stretch stimulates a dramatic increase in fibroblast collagen synthesis, deposition and reorganisation. Further to this, the data has demonstrated that load-induced collagen deposition in the two-dimensional model is mediated via the $\alpha2\beta1$ integrin receptor. This information has a number of potentially important implications in understanding both responses to mechanical forces *in vitro* and scar formation following dermal wound healing.

The question of how specific integrins regulate specific cellular responses despite binding the same substrate is very poorly understood. As discussed in Chapter 6, whilst it is known that different integrins have affinities for different matrix molecules, the reason for multiple integrins binding and signalling from the same substrate is under debate. $\alpha1\beta1$ and $\alpha2\beta1$ integrins both bind collagen, and data presented in this thesis demonstrating distinct roles for each receptor would disagree with the theory that this represents an example of redundancy in nature. Therefore, specific integrins must stimulate distinct signalling responses and thereby regulate an individual pathway.

$\alpha1\beta1$ is reported both in this thesis and other studies (Klein *et al*, 1991; Gardner *et al*, 1999) to play a key role in regulating collagen synthesis, both *in vitro* and *in vivo* in the context of dermal wound healing. The $\alpha2\beta1$ integrin is also very important in the deposition of excessive collagen, specifically in response to mechanical load. There are a number of models currently described in the literature, which attempt to explain the mechanism of mechanotransduction. These models are outlined in section 1.5.6 in the introduction to this thesis. The data presented in this thesis can now be

incorporated in the context of these mechanotransduction models. This and previous studies conducted in the two-dimensional mechanical loading system demonstrate a definite role for integrins in the transduction of a mechanical signal into a biochemical one (Wilson et al, 1995; MacKenna et al, 1998). The involvement of integrins agrees with the large majority of the currently hypothesised models of mechanotransduction. The study presented in this thesis is very important as it confirms the role of integrins in regulating mechanically induced protein synthesis within a cell, which in turn has dramatic biochemical and phenotypic consequences.

Furthermore, data in this study demonstrating changes in integrin cell-surface expression in response to stretch suggest that forces generate an alteration in the cell-surface receptor composition first, and this then allows the transduction and eventual conversion of this signal to an increase in collagen deposition. As the cytoskeleton is required to be intact for recruitment of some integrins (Vekemann et al, 1993; Carvalho et al, 1995) this suggests that links directly to the cytoskeleton could be responsible for the changes in integrin levels at the cell surface. It would appear that no signal to the nucleus or new transcription is required for this effect to occur, therefore it is feasible that mechanics could be responsible directly for this initial change in cell surface receptor levels.

The timing of events in 2-dimnesional loading system can be explianed in terms of control of regulation of both integrins, PCP and procollagen in response to mechanical load. The list below summarises the course of events involved in mechanical regulation of these factors, and the role of each molecule involved. These findings are also summarised in Fig 8.1.

Time	Response to mechanical load
16 hours:	increase in cell-surface $\alpha 2$ integrin subunit;
	Role: *regulates load-induced PCP and insoluble collagen deposition*
24 hours:	increase in PCP mRNA and protein;
	Role: *regulates load-induced deposition of procollagen*
	increase in cell-surface $\alpha 1$ integrin subunit;
	Role: *regulates growth factor-induced procollagen synthesis*
48 hours:	increase in procollagen mRNA and protein;
	increase in insoluble collagen deposition

Clearly, the responses to mechanical load occur in a regimented order, to achieve tightly regulated collagen deposition after 48 hours. In the 3D loading system, as reported in Chapter 7, collagen synthesis, deposition and fibril alignment in response to load occurs by 24 hours. The accelerated response in this system as compared to the 2D system may be a function of the collagen already present in the 3D gel and the regulatory effects that this existing collagen has upon the fibroblasts. It is possible that the upregulation of integrins in response to stretch in the 3D system is more rapid, and therefore the ensuing increase in PCP activation and collagen deposition is greatly accelerated. The density of the collagen surrounding the fibroblasts may govern this initial upregulation of integrins, clearly a key step in the formation of excessive collagen matrix. This would explain why in the 2D system, cells must synthesise collagen in the first 24 hours of culture in response to growth factors in order to respond to load, whereas in the 3D system, this collagen is already evident. Similarly,

it is possible that the collagen density surrounding fibroblasts also partially governs the prevention of further responses to load after 72 hours. The temporal responses of integrins to 3D mechanical load must be examined to begin to investigate this theory.

The concept of integrin involvement in mechanotransduction agrees with a number of currently proposed models, as described in section 1.5.6 of this thesis, including the tensegrity and three-layered theories. These models argue that connection of integrins to the cytoskeleton generates a direct mechanical link from cell-surface to nucleus, allowing rapid transduction of force and thus a fast, efficient load response. This model forms the basis of the stretch response mechanism in endothelial cells, as has been shown by magnetic twisting of integrin subunits using matrix-covered beads (Wang *et al*, 1993). Although the involvement of the cytoskeleton has not been assessed directly in the responses to load reported in this thesis, preliminary data from other members of the laboratory suggests that load responses are inhibited using cytochalasin D. This implies that both integrins and intact cytoskeleton are required for increased collagen deposition in this system.

These theoretical mechanotransduction models may also support the concept of synergy between growth factors and mechanical load, evident in both 2D and 3D loading systems. As previously discussed in chapter 3, growth factors are required for the mechanically induced increases in collagen deposition after 48 hours, but the mode of action of this synergy is poorly understood. It is possible that growth factor receptors may be upregulated by load to promote the enhanced procollagen synthesis, in the same way that integrin subunit expression is altered by stretch. The delay in the detection of increased procollagen synthesis in response to mechanical load is

consistent with the prior stimulation of the synthesis of other proteins, and this may include the production of growth factor receptors. This would also explain why conditioned media from stretched cells does not enhance procollagen synthesis in fresh fibroblast cultures.

It is evident that integrins are also key to the transduction of load and subsequent increased collagen deposition, and as load cannot stimulate a response in the absence of growth factors, it is likely that the signalling from both receptors types converge at a point intracellularly to regulate the response. As described in Chapter 6, numerous signalling pathways have been shown to be stimulated in response to mechanical forces, but the exact molecules involved in the synergy response are unknown. The MAP kinase pathways are certainly widely implicated in load responses (MacKenna *et al,* 1998) and the increase in phosphorylation of FAK and associated adapter proteins (Yang et al, 1997) may be responsible for this activation of signalling cascades. Current studies are also being undertaken to investigate the presence of load-responsive elements in the promoters of matrix and growth factor genes. It is possible that the convergence occurs at a transcriptional level, thereby allowing tight control over the final response to load.

In summary, therefore, it can hypothesised that there are three potential mechanisms by which load can regulate collagen deposition by acting in synergy with growth factors.

1. Growth factors stimulate the synthesis and deposition of collagen, which is required for cellular detection of mechanical load.

2. Growth factors are required for the upregulation of integrins α1β1 and α2β1. The integrins are then available for recruitment to the cell surface to act as mechanotransducers.

3. Integrin and growth factor signalling pathways converge (or are amplified) either at a cytoplasmic level or a transcriptional level, acting to upregulate the collagen gene directly, or genes involved in further mechanotransduction responses.

It is certainly possible that all three of these mechanisms occur within a cell subjected to mechanical forces. Similarly, there are likely to be other proteins, possible even matrix molecules, which are regulated in some context by load, and these processes may also be controlled by similar mechanisms. The exact signalling cascades stimulated in response to stretch and the purpose of the activation are still largely under investigation. However, with the increasing number of *in vitro* loading systems available, knowledge of the numerous fascinating cellular responses to mechanical forces is expanding rapidly.

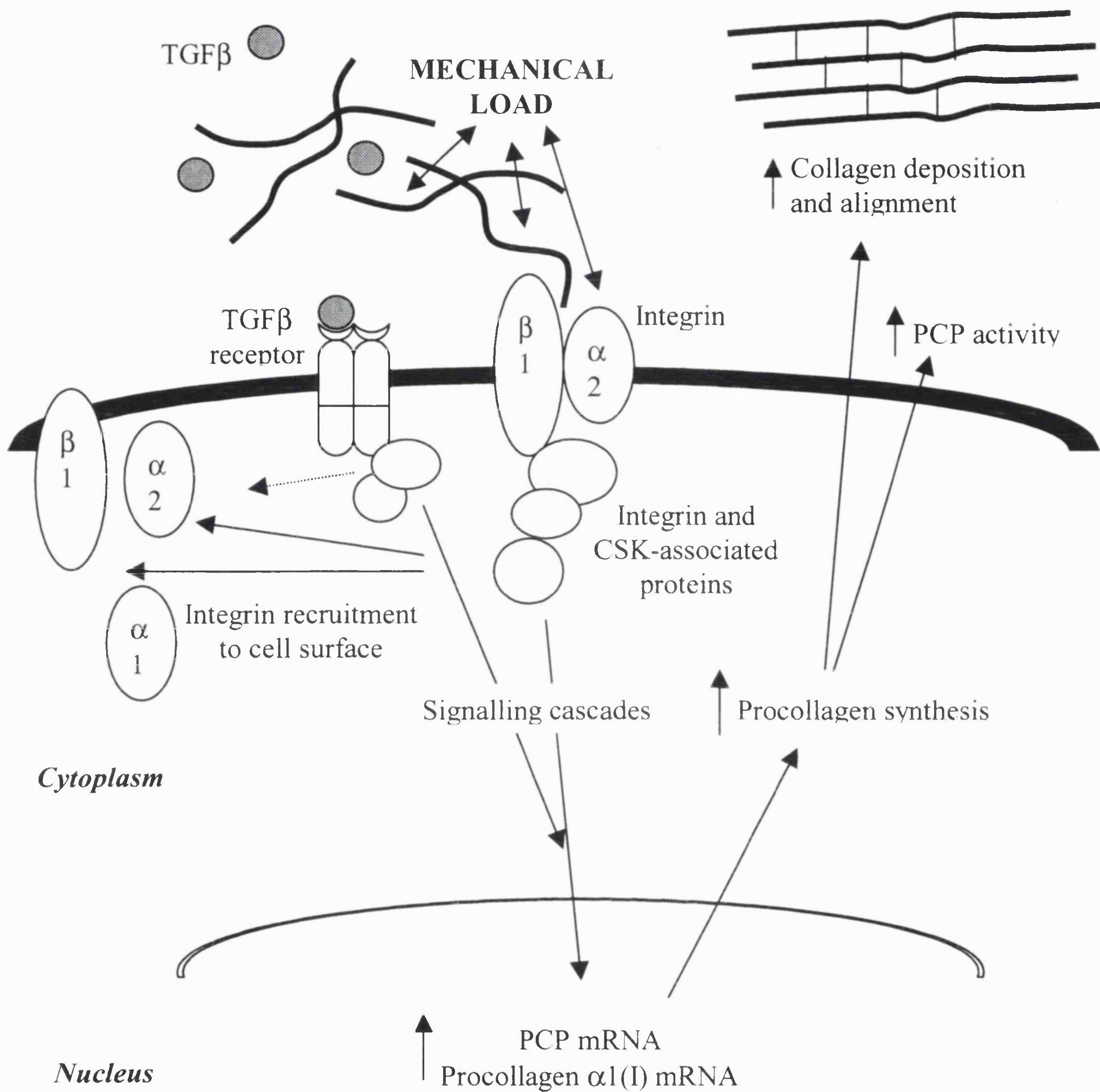


Fig. 8.1: diagrammatic summary of thesis findings

The regulation of collagen deposition by mechanical load. Growth factors act via respective receptors to stimulate the synthesis of procollagen and integrins subunits. Mechanical load stimulates the recruitment of integrins α1β1 and α2β1 to the cell surface. These receptors are in turn mechanosensitive, and stimulate signalling cascades which either alone or in combination with growth factors, cause the upregulation of PCP mRNA and protein synthesis. This is followed by an increase in procollagen mRNA and protein synthesis, which then is exported from the cell and is processed into insoluble collagen by the enhanced levels of PCP. The surrounding mechanical forces then align the deposited collagen.

8.1.2 Comparison of in vitro loading systems

The studies in this thesis were conducted in two different *in vitro* mechanical loading systems. The use of a both a 2-dimensional (2D) and 3-dimensional (3D) system allowed a wide range of studies to be undertaken to investigate cellular responses to load. Whilst each system was appropriate for some studies, they were not both appropriate for every investigation for a number of reasons.

Firstly, the 2D system, which loads cells as a monolayer was very convenient for the study of both collagen synthesis and integrin expression. This is because the cells are loaded in 6-well plates which makes harvesting of cells and protein for biochemical analysis very precise and convenient. The 2D system is also very convenient for immunocytochemical analysis as the cells are easy to biologically fix within the plates, and are relatively easy to visualise by microscopy. However, there are two main drawbacks to using the 2D device. However, due to the nature of the loading applied the cells are not all subjected to an equal amount of strain. Cells 3mm in from the edge of the well experience the most load, and this decreases as a gradient into the centre of the well, where none of the cells are loaded. Whilst this differential strain is calculated and is unimportant when entire wells are harvested for biochemical analysis, it does not allow precise visual assessments of cell responses to load. Secondly, as cells are plated as a monolayer, and therefore only experience loading at the cell-elastomer contacts, it has been argued that this system is not phisiologically appropriate. However, it is an excellent system for the analysis of basic cellular responses to forces, particularly protein synthesis and signalling.

The 3D loading system, which applies a uniaxial load to cells within a collagen gel, was used in this thesis for studying collagen synthesis and fibril alignment in response to forces. As with the 2D device, this 3D system has a number of advantages and disadvantages associated with it. Firstly, the system is a 3-dimensional system, where cells are exposed to matrix from all angles, and forces from all angles. This is a great advantage as it allows extensive analysis, both at electron and light microscopy levels, of cellular infrastrucutre and matrix changes in response to load. It has also been argued that cells within a 3D matrix are in more physiological conditions as compared with those in a monolayer, and therefore data obtained in this system is more relevant to a true *in vivo* situation. Similarly, the system is easily manipulable and flexible in terms of changing the nature of the 3-dimensional matrix in which the cells are embedded. For instance, the addition of fibronectin or fibrin matrices into the collagen lattice and then assessing responses to load would provide a more accurate representation of a wound environment for the study of these processes. However, this device also has drawbacks. As the cells are embedded in a gel, retrieving them for biochemical analysis is relatively difficult, as is the analysis of cellular protein synthesis as the proteins become trapped within the lattice. Similarly, the analysis of phosphorylation or levels of short half-life signalling molecules is very difficult as harvesting cells from gels is often very time-consuming.

These different devices have been employed for specific biochemical and morphological analyses in this thesis, demonstrating that each system has its benefits and pitfalls. However, the development of more sophisticated *in vitro* devices will allow the analysis of mechanisms of mechanotransduction in one universal system.

8.1.3 The in vivo *and clinical implications of this thesis*

To extrapolate to an *in vivo* pathological scenario of wound healing, the fibroblasts are constantly being exposed to extraneous mechanical loading, as well as to growth factors released during the inflammatory response. In dermal wound healing, the final output is in the form of a visible scar, which is a reorganised network of collagen fibres, laid down in an ordered fashion unlike the normal skin in which collagen fibres are disorganised. This being the case, the final scar morphology is very dependent on the amount of collagen laid down and any subsequent remodelling and reorientation that takes place. The data in this study demonstrates that mechanical loading of the fibroblasts involved in a wound healing response may be critical in defining these factors. The synergistic effect of load and growth factors acts upon the cells to stimulate mRNA synthesis and the release of enzymes, which in turn affect the production and deposition of new matrix. This again is regulated externally by the amount and type of matrix that the cell is embedded in. This may also be an important factor in governing timing of response in a wound-healing situation.

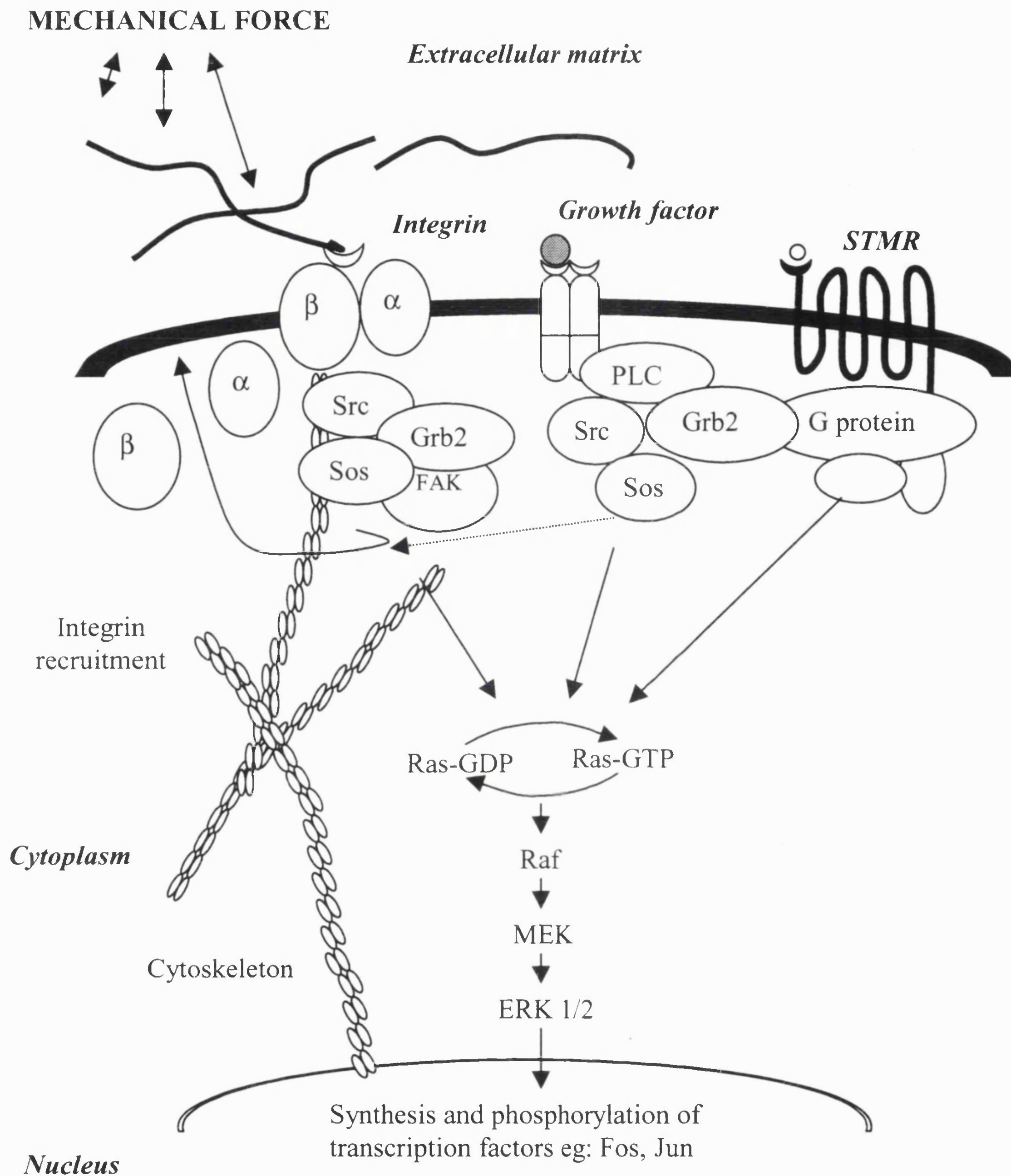
The current hypothesised model of mechanotransduction is shown in figure 8.2. Theoretically, there are a number of different ways in which deleterious responses to mechanical load can be manipulated. Clinical studies have already attempted to reduce the loading around a healing wound in order to reduce scar size, which would appears to be an effective tool. However, total anti-scarring therapies may involve both manipulation of mechanical load physically, and of the factors released in such an environment. Based upon this study, one of these factors would appear to be PCP, the inhibition of which would prevent the excessive deposition of collagen. However, it

may be that inhibition of PCPE is more effective as this will merely reduce the activity of PCP, rather than totally depressing the activity, which may in itself prove to be deleterious.

Integrins are also potentially very good therapeutic anti-scarring targets. As data in this thesis has demonstrated, both $\alpha1\beta1$ and $\alpha2\beta1$ are important in regulating collagen deposition by human dermal fibroblasts, but $\alpha2\beta1$ seems to specifically control excessive load-induced insoluble collagen formation. For this reason, $\alpha2\beta1$ represents a very good therapeutic target alone as this receptor is involved in the processing of excessive procollagen, and therefore inhibition of this receptor would theoretically not affect growth factor stimulated collagen deposition and the integrity of the final scar. Importantly, the role of $\alpha2\beta1$ in scarring is supported by *in vivo* studies. Investigations have shown that while $\alpha2\beta1$ is hardly detectable in normal pigskin and in wound granulation tissue when matrix is still rich in fibronectin, its expression is strongly upregulated with the formation of collagenous scar and the beginning of scar contraction (Xu *et al*, 1996). Inhibition of $\alpha2\beta1$ would also theoretically inhibit excessive PCP production, and thereby negate the need for a PCP or PCPE inhibitor. Integrin inhibitors are currently in development for cancer therapy purposes, and it is possible that these antagonists may be directed towards anti-scarring and anti-fibrotic agents in the future.

In conclusion, mechanical load appears to be an important factor governing collagen synthesis, processing and organisation by human dermal fibroblasts. This may have

implications in the development of therapeutic agents designed to combat pathological situations involving fibrosis and scarring.



8.2 Proposed model of mechanotransduction

A hypothetical model of mechanical transduction. Growth factors and mechanical load act to upregulate the recruitment of integrins to the cell surface. These integrins then transduce the mechanical signal to the nucleus via linkage to the cytoskeleton, FAK and a downstream signalling cascade, such as the MAPK cascade. Growth factor receptors also stimulate signalling cascades which converge with integrin signalling to stimulate transcription factor translocation to the nucleus. These factors then act directly upon load response elements within promoters of specific genes, or stimulate the upregulation of load-responsive factors.

8.2 FUTURE DIRECTIONS

The findings from this thesis may be furthered in a variety of novel studies. The finding that PCP is upregulated an *in vitro* system of mechanically loading dermal fibroblasts, and that this is regulated through a specific integrin, is very important in the understanding of mechanisms of collagen deposition. This mechanism and indeed mechanisms involved in basal insoluble collagen fibril formation are poorly understood.

The mechanism of feedback regulation of collagen synthesis and PCP expression in response to mechanical load requires in-depth investigation. The regulation of load-induced PCP via integrin $\alpha2\beta1$ may be very important in the overall regulation of procollagen synthesis and deposition. The potential binding and activation of PCP by $\alpha2\beta1$ also requires investigation to assess the direct role of integrins in load-induced gene and protein regulation. Co-precipitation studies to investigate the potential binding of procollagen and PCP to $\alpha2\beta1$, in conjunction with histological localisation studies, would also begin to address the direct role of this receptor in collagen fibril formation. The elucidation of the specific signalling pathways or transcription factors involved in the load-induction of procollagen synthesis and PCP is also vital to assist in the understanding of responses load.

These studies may be furthered, by investigating other known pro-fibrotic factors in this *in vitro* loading system, such as ascorbate, other TGFβ isoforms and the reported TGFβ mediator connective tissue growth factor (CTGF), and the role these play in

controlling collagen deposition and PCP expression in loaded and unloaded fibroblasts. Similarly, the effects of mechanical load on receptors for these growth factors would also be of great interest and may begin to examine the synergy of polypeptides such as these and mechanical tension.

It would be fascinating to test the role of $\alpha 2\beta 1$ in transducing load-induced collagen deposition and alignment in the 3D system used in this thesis. Reproducing these experiments in a more "physiologically relevant" system such as the collagen gels would provide even more compelling evidence that this mechanism is important *in vivo*. The newly developed *in vitro* dermal equivalents may also provide a very useful tool to assess the effects of the 3-dimensional loading system on fibroblast activation. Dermal equivalents such as the commercially available Apligraf (Organogenesis Inc.) incorporates allogeneic cultured human skin cells in a full-thickness skin construct containing both epidermal and dermal layers (Sabolinski et al, 1996). There is a differentiated epidermal layer composed of living human keratinocytes; the dermal layer is formed of living human fibroblasts. The application of mechanical load to wounded living skin equivalents would provide a convenient *in vitro* system to test the concepts discussed in this thesis. As controlled forces can be applied in the TCFM, the effects of differing loads on matrix deposition and alignment could be easily investigated both histologically and using radioactively labelled isotopes. Using a system encompassing both epithelial cells and fibroblasts together also allows the analysis of "cross-talk" between these two cell types in response to stretch during tissue repair. Keratinocyte responses to load, a currently under-researched area could also be addressed in this system. The use of inhibitors and antisense oligonucleotides

to inhibit individual growth factors may assist in the identification of load-induced paracrine-acting factors.

Clearly, testing these concepts in an *in vivo* model of wound healing, for instance excision wounds in rat or pigskin, would provide conclusive evidence of the role of mechanical forces in dermal scarring. Devices to reduce tension around the healing wound could be used either alone or in conjunction with biological inhibitors of PCP or integrin receptor activation to achieve a combination therapy effect.

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PUBLICATIONS ARISING FROM THIS THESIS

Parsons M, Kessler E, Laurent GJ, Brown RA and Bishop JE (1999) Mechanical load enhances procollagen processing in dermal fibroblasts by regulating levels of procollagen C-proteinase. *Exp Cell Res;* 252 (2) p319-331

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