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Strategies to optimise angiogenesis in Synthetic Dermal Equivalents using *in-vitro* models

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Abstract

Large burns necessitate skin cover. Skin grafts provide a simple solution but are limited in availability. Cultured keratinocytes provide epidermal cover but invariably fail unless grafted on dermis. Synthetic Dermal Equivalents (SDEs) are mostly collagen based and are dogged by poor take. This thesis concentrates on enhancing the angiogenic capability of SDEs to increase take.

Aims;

- To develop components for a "smart" pro-angiogenic matrix for reliable take.

- To explore and investigate clinical adjuncts, to increase graft angiogenesis.

- To evaluate angiogenic factors and extracellular matrix components for endothelial cell migration promotion to include in a potential skin equivalent.

- To explore the *in-vitro* angiogenic properties of SDEs and the role/effective regimes of Ultrasound and Topical Negative Pressure (TNP).

Greatest growth factor stimulation was from TGFß. Influence of migration by angiogenic factors was minimal compared to fibrin. Basal invasion into collagen was low. Fibrin stimulated migration seven fold over that of collagen ($p<0.01$ ANOVA). Fibrin subunits had equal effect to fibrin. Glycosaminoglycans, Fibronectin and Vitronectin addition increased migration over that of collagen. The addition of collagen to fibrin inhibited invasion. Ultrasound at an optimum energy of $0.8W/cm^2$ gave a 3 fold increase in migration over control conditions. This was equal to the addition of fibrin. Angiogenesis induced by TNP was optimum in Integra using an intermittent regime.

This thesis has shown that collagen, the principal component of all SDEs fails to provide an optimal matrix for invasion of endothelial cells. We have found that the most favourable matrix constituents for endothelial cell migration are fibrin and fibrinogen with fibrin exerting a sevenfold increase in endothelial cell migration over collagen. It has also shown that TNP provides equal endothelial cell ingress to both US and fibrin, only when used intermittently with Integra.

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Abbreviations

ACE	Angiotensin Converting Enzyme
ALK	Activin Receptor-Like Kinase
AmPs	Ammonium Persulphate
Ang	Angiopoietin
ANOVA	Analysis of Variance
bFGF	Basic Fibroblast Growth Factor
Bcl	B cell lymphoma
BS	Bovine Serum
CAM	Chorioallantoic Membrane
CD44	Cluster of Differentiation antigen 44
CO_2	Carbon Dioxide
COX	Cyclooxygenase
CYT C	Cytochrome C
DNA	Deoxyribonucleic acid
DPX	Distyrene Plasticizer Xylene
DSE	Dermal Skin Equivalent

EACA	ε—Aminocaproic acid
ECGF	Endothelial Cell Growth Factor
ECGS	Endothelial Cell Growth Supplement
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
eNOS	endothelial Nitric Oxide Synthase
FBS	Foetal Bovine Serum
FCS	Foetal Calf Serum
FDP	Fibrin Degradation Products
FGF	Fibroblast Growth Factor
Flt	Fms-Like Tyrosine kinase
GAGs	Glycosaminoglycans
GEC	Gel Exclusion Chromatography
GlcNAc	N-acetylglucosamine
GalNAc	N -acetylgalactosamine
HBSS	Hanks' Balanced Salt Solution

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HEPES	Hydroxyethyl-1-Piperazineethanesulfonic acid
HGF	Hepatocyte Growth Factor
HIF	Hypoxia Inducible Factor
HIV	Human Imunodeficiency Virus
HLA DR	Human Leucocyte Antigen D Region
HMVEC	Human Microvascular Endothelial cells
HPMEC	Human Placental Microvascular Endothelial cell
HUVEC	Human Umbilical Vein Endothelial Cells
IGF	Insulin like Growth Factor
IL	Interleukin
KCI	Kinetic Concepts, Inc
KDR	Kinase insert Domain containing Receptor
KGF	Keratinocyte Growth Factor
LMP	Lower Melting Point
MCP	Monocyte Chemotactic Protein
MM	Minimal Medium

MMP	Matrix Metalloproteinase
mRNA	Messenger Ribose Nucleic Acid
MT-MMP	Membrane Type - Matrix Metalloproteinase
NO	Nitric Oxide
PA	Plasminogen Activator
PAI	Plasminogen Activator Inhibitor
PAL-E	Pathologische Anatomie Leiden-Endothelium
PAR	Protease Activated Receptor
PBS	Phosphate Buffered Saline
PD ECGF	Platelet Derived Endothelial Cell Growth Factor
PDGF	Platelet Derived Growth Factor
PDP	Platelet Depleted Plasma
PECAM	Platelet Endothelial Cell Adhesion Molecule
PKC	Protein Kinase C
PMA	Phorbol β-Myristate Acetate
PI3K	Phosphoinositide-3 Kinase

PlGF	Placental Growth Factor
PTFE	Poly Tetra Fluoro Ethylene
RAFT	Restoration of Appearance and Function Trust
RGD	Arginine Glycine Aspartate
SDE	Synthetic Dermal Equivalent
SDR	Synthetic Dermal Replacement
SDS PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SIKVAV	Ser-Ile-Lys-Val-Ala-Val
TBDN	Tubedown
TGF	Transforming Growth Factor
TIMPS	Tissue Inhibitors of Metalloproteinases
TNF	Tumour Necrosis Factor
TNP	Topical Negative Pressure
TPA	Tissue Plasminogen Activator
TPA	Tetradeconyl Phorbol 13 Acetate
UK	United Kingdom

UPA	Urokinase Type Plasminogen Activator
US	Ultrasound
USA	United States of America
UV	Ultra-Violet
UW	University of Wisconsin
VAC	Vacuum Assisted Closure
VE-cadherin	Vascular Endothelial - cadherin

Chapter 1

General Introduction

Advances in biotechnology and bioengineering have given Burns and Plastic surgeons the potential to treat extensive tissue loss. The newly developed replacement "skins," whilst providing hope, are failing to deliver reliable performance and have failed to transform patient care and outcome as initially expected (Bar-Meir, Mendes et al. 2006). This thesis aims to improve the care of patients suffering from large wounds, such as burns, in providing a novel approach to improve the performance of a second generation dermal replacement by the incorporation of angiogenic components or correct use of mechanical adjuncts to improve the "take" of synthetic skins. In order to survey the issues involved, this chapter aims to explain the involved skin anatomy, available skin replacements, introduce the central hypothesis to the thesis and give the reader an explanation of angiogenesis to help understand the angiogenic methods involved in increasing the take of dermal replacements.

1.1 Skin Anatomy

In addition to its obvious property of enveloping the body, the skin has a wide range of diverse functions including protection against injury, thermoregulation, waterproofing and fluid conservation. It is of considerable importance in the absorption of ultraviolet radiation and in the production of vitamin D; it acts as a barrier to pathogenic organisms and functions in the detection of sensory stimuli.

The skin conveniently divides into two distinct layers, the Epidermis and its appendages, derived from Ectoderm, and the Dermis with the underlying subcutaneous fat derived from Mesoderm (the nerves and Melanocytes are of neuroectodermal origin, figure 1.1). The epidermis is a multilayered (stratified) squamous epithelium from which arise the pilosebaceous follicles, apocrine glands and eccrine sweat glands. The dermis consists of the ground substance plus a fibrous component (collagen and elastin).

There is considerable regional variation in skin structure and to some extent function. Skin is divided into two types: glabrous and hairy. Glabrous skin (typified by a thick keratin layer) covers the surfaces of the palms and soles, while hairy skin covers the rest of the body. Hair production is maximum about the head, the axilla and pubic regions and on the face of males. Sebaceous glands are especially numerous about the face and nose, whereas eccrine glands are most commonly found on the palms and soles. The surface of the skin is far from regular, being marked by a series of complex creases determined by

the underlying epidermal ridge pattern. This is clearly demonstrated by the whorls, loops and arches that constitute the fingerprints. Mucous membranes differ from skin by the absence of both granular and horny layers.
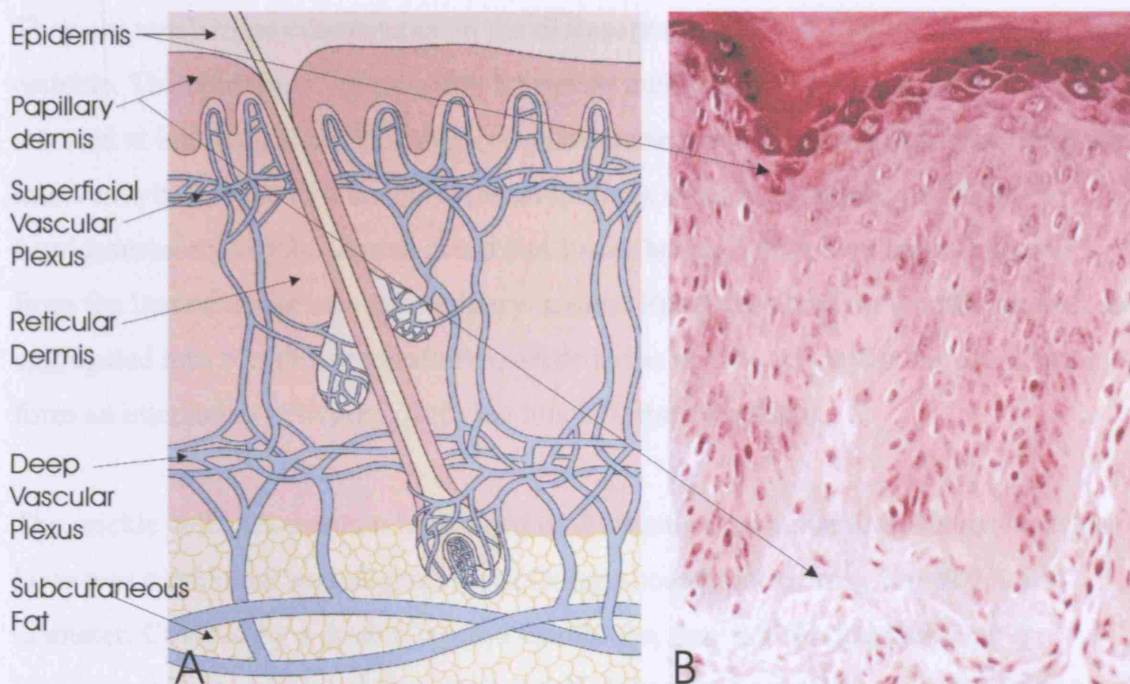
Figure 1.1 The relationship of Epidermis to Dermis, showing the prominence of the border between the two, the epidermal ridge as well as associated vascular plexuses (taken from the Atlas of Clinical dermatology, (Du Vivier 2002))

1.1.1 The epidermis

Histologically, the epidermis consists of at least four cell types (Keratinocytes, Melanocytes, Merkel cells and Langerhans' cells) and has four clearly defined layers: the basal cell, prickle cell, granular cell and keratin layers. A fifth layer may be interposed between the granular and keratin layers on the palms and soles. The basal cell layer is the germinative layer of the epidermis. With each division, approximately 50% of the daughter population contributes to the developing epidermis. It is thought that the epidermal transit time is approximately 30 days. Maturation consists of the conversion of columnar basal cells into the fully keratinised cells of the epidermal horn and involves a transformation of cellular polarity, basal cells being arranged at right angles to the basement membrane while the cellular residues of the keratin layer lie parallel.

These perpendicularly orientated columnar cells have basophilic cytoplasm and round to oval hyperchromatic nuclei; when mature they acquire the polyhedral outline of the

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prickle cell layer. The accumulation of keratohyalin granules characterises the granular cell layer. Further maturation leads to loss of nuclei and flattening of the cellular outline until flattened plates of the keratin layer are fully formed. The keratinocytes are adherent with each other by intercellular bridges (desmosomes) best seen in the prickle cell layer. They are much more conspicuous in the disease states of the skin involving intercellular oedema. The epidermis lies on a thin basement membrane (figure 1.2). Hemidesmosomes are seen at intervals along the basal cell plasma membrane and beneath this a clear zone intervenes before the lamina densa (basal lamina). Anchoring filaments adjoin the hemidesmosomes to the lamina densa and its anchoring fibrils may be seen spreading from the lamina densa into the papillary dermis. Basal cells contain tonofilaments loosely aggregated into bundles or tonofibrils, while in the prickle cell layers the tonofilaments form an interlacing network occupying much of the cytoplasm.

The prickle cells also contain lamellated oval structures containing lipids and enzymes known as membrane coated granules or Odland bodies, measuring 100-500 nm in diameter. Covered by a double layered membrane, they contain parallel lamellae approximately 2 nm thick that are orientated along the short of the granule. Odland bodies are located particularly in the region of the plasma membrane. In addition to the membrane coating granules, the granular cells contain keratohyalin granules, which are not membrane bound and consist of closely packed amorphous particles. The cells of the keratin layer contain tonofilaments embedded in an amorphous matrix and are characterised by a thickened cytoplasmic membrane. The intercellular spaces contain a lipid material probably derived from the membrane coating granules which functions as an intercellular cement.

Tonofilaments and keratohyaline granules are largely composed of protein, while membrane coated granules contain large amounts of lipids and hydrolytic enzymes (possibly involved in exocytosis of the granules).

Keratinization is the process of epidermal differentiation by which basal cells are converted into the protective membranous horny layer. Its exact mechanism is not fully understood, but it depends on the development and interplay of the three intracellular organelles, namely tonofilaments, keratohyalin granules and membrane coated granules.

Maturation of the epidermis involves an increase in the number of tonofibrils followed by their incorporation into the amorphous substance of the keratohyalin granules. There is some evidence suggesting that desmosomes play an important role in keratinisation, possibly functioning as attachment sites for tonofilament orientation. The cells of the horny layer are cemented together to form a tough and flexible membrane, the superficial aspect, which is continuously being shed as large clusters of squames. The keratin layer contains lipid deposits between the horny cells that prevent the loss of body fluids and influx of water into the skin. The strength and integrity of the horny layer is believed to result from the presence of disulphide bonds between adjacent keratin molecules.
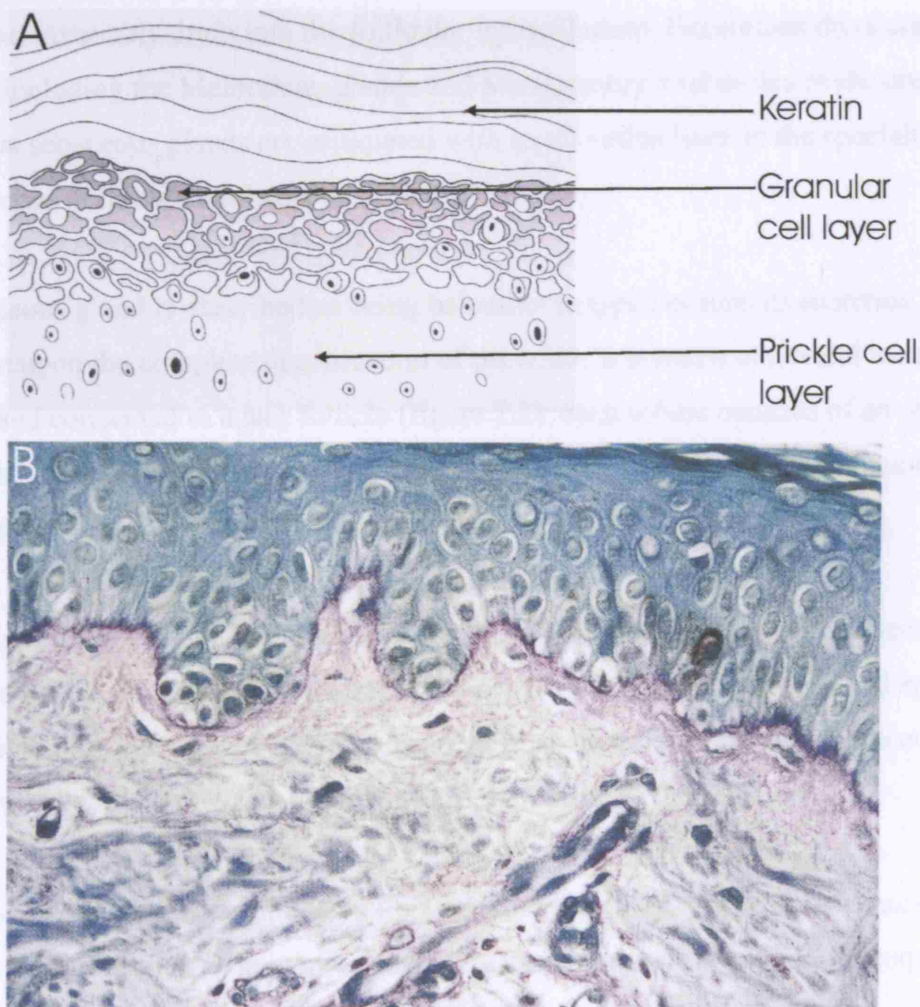
Figure 1.2 The layers of the epidermis shown diagramatically "A" and the prominence of the basement membrane shown histologically "B" (purple staining), (Du Vivier 2002).

1.1.1.2 Epidermal appendages

1.1.1.2.1 Sebaceous glands

The sebaceous gland arises as a lateral protrusion of the outer root sheath of the developing hair follicle. It can be first clearly identified at 13-15 weeks of gestation. During foetal life, its secretary product, sebum, is partially responsible for the vernix caseosa, the other constituents being foetal hair and squames. The sebaceous gland is largely inactive during prepubertal life but enlarges and becomes functionally active during and after puberty. While fairly widespread in distribution, sebaceous glands are not found on the palms or soles but are concentrated about the face and scalp, in the midline of the back and in the perineum. As can be imagined from their development, they almost invariably drain into the follicular infundibulum. Exceptions do occur, however, including the Meibomian glands and Montgomery's tubercles of the areolar. The largest sebaceous glands are associated with small vellus hairs in the specialised pilosebaceous units called sebaceous follicles.

The sebaceous gland is described as being halocrine in type because its secretion is dependent upon the complete degeneration of the acini. It consists of several lobules lying adjacent and connected to a hair follicle (figure 1.3); each lobule consists of an outer layer of cuboidal basophilc cells from which arises the inner zone of lipid laden vacuolated cells. Its secretions drain into the sebaceous duct.

The mechanism of control of sebaceous activity is incompletely understood. Secretion appears to have a circadian rhythm, largely under the control of androgens and appearing to be inhibited by oestrogens. It comes as no surprise, therefore that male sebaceous glands are larger and more functionally active than those of the females

Sebum is an exceedingly complicated lipid mixture including triglycerides, wax esters and scalene. Its function in humans, although uncertain, possibly includes waterproofing, control of epidermal water loss and a protective function inhibiting the growth of fungi and bacteria.

1.1.1.2.2 Apocrine glands

Apocrine glands are found predominantly in the anogenital and axillary regions. They are derived from the epidermis and develop as an out-growth of the follicular epithelium.

Their function is unknown, but in animals they are thought to be responsible for scent production. Similar to sebaceous glands, apocrine glands are rather small in childhood, becoming larger and functionally active at puberty.

Apocrine glands consist of two distinct components: a secretory component situated in the lower reticular dermis or subcutaneous fat and a tubular duct lining the gland with the pilosebaceous follicle at a site above the sebaceous duct. Microscopically, the secretory portion comprises an outer layer of myoepithelial cells and an inner layer of cuboidal to columnar eosinophilic cells (figure 1.3). The duct portion consists of a double layer of cuboidal epithelium. The mechanism of apocrine secretion and control of apocrine glands is uncertain but they do receive adrenergic sympathetic innervation (consider the increased secretion in response to fear). The unpleasant odour of apocrine secretion, which is itself odourless, is from breakdown products formed by the cutaneous bacterial flora.

1.1.1.2.3 Eccrine sweat Glands

Eccrine sweat glands derive from a specialised down-growth of the epidermis at about the fourth month of intrauterine life. They are found everywhere on the skin but are not present in the mucous membranes. Their sites of maximum distribution are the palms, soles, axillae and forehead. Histologically, they are divided into four subunits: a coiled sectretory gland, a coiled dermal duct, a straight dermal duct and a coiled intraepidermal duct. The secretory component lies in the lower reaches of the reticular dermis or around the interface between the dermis and subcutaneous fat. It consists of an outer layer of contractile myoepithelial cells and an inner layer of secretory cells (figure 1.3). The latter consists of two cell types: larger clear cells, which appear to be responsible for its watery secretions and a smaller darkly staining mucopolysaccharide containing cells. Between adjacent clear cells are canaliculi, which open into the lumen of the tubule. The dermal duct is formed from a double layer of cuboidal basophilic cells. The duct is not just a conduit but has a biologically active function in modifying the composition of eccrine secretion, in particular the reabsorption of water. The intraepidermal duct opens directly onto the surface of the skin. The function of the eccrine gland is under control of cholinergic post-ganglionic sympathetic nerves. The activity of the secretory component is stimulated by thermal, mental and gustatory functions. Thermal sweating is dependent on an intact hypothalamus (activated by temperature changes of its perfusing blood) Thermoregulatory sweating occurs especially on the face and upper trunk. Mental

sweating is presumably under the control of the limbic lobe. This induces particularly palmar sweating. Gustatory sweating of the lips, forehead and nose (as after a hot spicy meal) is of uncertain function and control. It is a particular complication of parotid gland resections. Sweat of eccrine type has a basic similarity to the plasma from which it is derived, the duct appearing to be largely responsible for the modifications that occur.
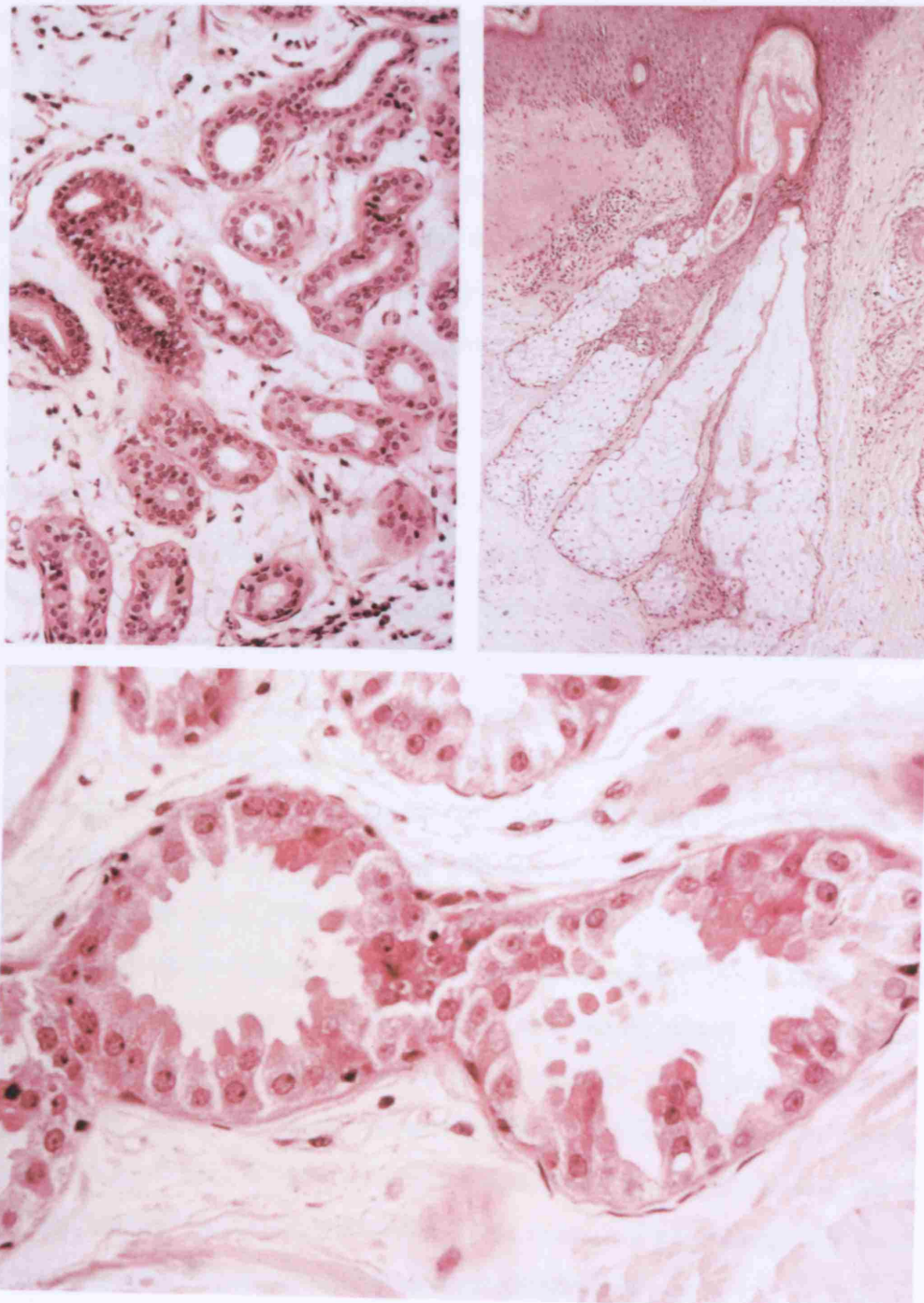
Figure 1.3 Histological slides of; an eccrine gland (top left), a sebaceous gland (top right) showing numerous lobules arising from a hair follicle and an apocrine gland (bottom) (Du Vivier 2002).

It is a clear hypotonic solution with a PH in the range 4-6.8. In addition to water, it contains sodium, chloride, potassium, urea and lactate.

1.1.2 The Dermis

The Dermis is the supporting layer of the epidermis and consists of a fibrous component (collagen and elastin) together with the so-called ground substance. Lying within it are the epidermal appendages, the neurovasculature and a cellular component including fibroblasts and inflammatory cells. The dermis is divided into two layers: the papillary (including a periadnexal component) and the reticular dermis. The papillary dermis is bounded superiorly by the epidermis, laterally by the epidermal ridges and inferiorly by the superficial vascular plexus and reticular dermis.

1.1.2.1 Collagen

Collagen gives the dermis its structural stability. In the papillary dermis, it consists of fine fibres in an ostensibly haphazard arrangement, while in the reticular dermis it consists of broad bundles lying roughly parallel to the epidermal surface. Collagen is formed within the ribosomes of fibroblasts; the essential subunit of collagen is the monomer tropocollagen, which has a molecular weight of approximately 300 000 and is composed of three peptide chains. Each chain has a helical structure and the three chains are intertwined to form a superhelical molecule. The structural stability of collagen is increased by intra- and intermolecular cross linkages, the latter including side to side, end to end and overlapping types. This produces an enormously strong fibrillary structure. Collagen characteristically contains the amino acids hydroxyproline and hydroxylysine. Collagen is not an homogenous entity but consists of a variety of genetically distinct subtypes: designated types I – XX according to morphology, amino acid composition and physical properties. In the dermis, the broader bands of reticular collagen are type I, the most common form, while the fibres (also known as reticulin) of the papillary dermis are type III. When longitudinal sections of collagen are examined microscopically they exhibit cross striations with a periodicity of approximately 64 nm.

1.1.2.2 Elastic tissue

Elastic fibres are intimately associated with collagen. In the papillary dermis, the elastic fibres are thin and tend to run at right angles to the skin surface, whereas those in the

reticular dermis are thicker and tend to lie parallel to the skin surface. Like collagen, elastic fibres are produced by fibroblasts. Ultrastructural examination shows elastic tissue to consist of an amorphous electron dense component (elastin), in which are embedded microfibrills. While elastic fibres are responsible for cutanoeus elasticity they are also thought (in combination with ground substance) to be responsible for prevention of overextension.
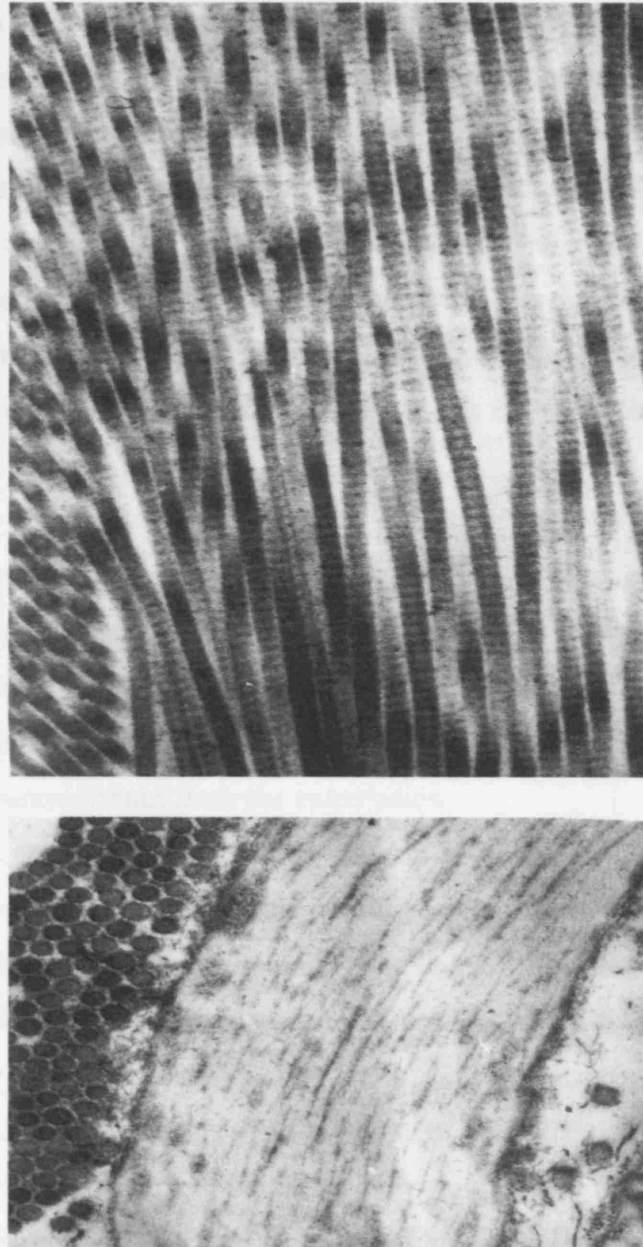
Figure 1.4 The typical cross striations of collagen (upper) and amorphous elastin (lower) shown with electron microscopy (x 47000 and x 19000 respectively) (Du Vivier 2002).

1.1.2.3 Ground Substance

Ground substance is another product of fibroblasts and accounts for a large proportion of the volume of the dermis. Ground substance is not merely an amorphous material in

which the fibrous components are embedded but can be best visualised as a gel-like substance existing in intimate chemical relationship with the fibrous components of the dermis. In addition to large quantities of water it consists of the glycosaminoglycans; hyaluronic acid, chondroitin 4 sulphate and dermatan sulphate.

1.1.2.4 Cutaneous Blood vessels

The skin receives an extensive vascular supply from vessels within the subcutaneous fat. From these arise two vascular plexuses linked by intercommunicating vessels; one, the deep vascular plexus, lies in the region of the interface between dermis and subcutaneous fat and the other, the superficial vascular plexus, lies in the superficial aspects of the reticular dermis and supplies the papillary dermis with a candelabra-like capillary loop system. Each loop consists of an ascending arterial limb and a descending venous limb. The collagenous component of the dermis receives only a limited supply, most of the capillary systems being associated with the metabolically active epidermis and its appendages.

A specialised cutaneous arteriovenous anastomsis, the Sucquet Hoyer canal is found in the dermis of the fingertips and to a lesser extent elsewhere on the body. The canal is surrounded by several layers of modified smooth muscle cells that function as a sphincter. These anastomoses enable the capillary networks of the superficial dermis to be bypassed, thus increasing the venous return from the extremities.

Cutaneous blood flow (under hypothalamic control) is of extreme importance in thermoregulation. Mediated by the sympathetic nervous system, heat loss can be increased or diminished by varying the volume of blood entering in an increased blood flow to the papillary dermis, accompanied by an increase in eccrine sweat gland secretion. Evaporation of sweat cools the outer parts of the body, with a resultant diminution of temperature of circulating blood. Thus temperature control depends upon a delicate interplay between vascular and sweat gland function. The dermis also contains an extensive lymphatic system closely associated with the vascular plexuses.

1.2 Management of the burn wound

In the UK 250,000 people are burnt per annum, 14,000 of which need specialist medical attention (Committee 2001). At least 1% of patients admitted to hospital die from their

burns (Committee 2001). Despite advances in burn and intensive care the mortality rate of such injuries in the elderly and young has not dramatically decreased in the past two decades. One of the improvements in burn treatment has been the creation of specialised burn units/centres. Prior to this patients were managed in general surgical departments with inadequate and conservative treatments often confined and neglected due to their "foetid odour" (Monafo 1992). The second world war produced a huge number of burns victims and with it invaluable experience in the treatment of large wounds. This brought advances in the treatment of burn shock, pulmonary injury, suitable resuscitation, limitation of infection and the early excision of burns tissues and surgical scar reduction. Indeed the main cause of death from burns in the 1940s and 50s was inadequate fluid resuscitation in the first hours after injury, as opposed to infection. In the 1960s and 70s with advances in fluid resuscitation, wound sepsis became the leading cause of death.

Figure 1.5 Bilateral circumferential lower limb burns following fascial excision

Improvements in resuscitation have left the surgeon with larger wounds for reconstruction in physiologically stable patients. These wounds provide large amounts of dead necrotic tissue in contact with healthy or salvageable tissue. This eshar can be left to autolytically debride, but this goes against many of the early surgical principles of debridement (Paré). Excision of such tissue has only become routine after the development of techniques in critical care, blood banking, anaesthesia and the ability to rapidly harvest and mesh skin to ensure graft survival. However with the advent of advanced antibiosis in the 1960s many burns centres chose to avoid early excision and grafting. Many centres chose to use

silver sulphadiazine as a topical antimicrobial agent, this treatment although without metabolic problems did not decrease the overall burns mortality. Moreover long term follow up showed that the risk of hypertrophic scarring was far greater with such conservative care.

Janzekovic published a paper in the 1970s that advocated initial debridiment in a tangential fashion until a viable vascular bed was reached (figure 1.5) and then the subsequent wound was grafted with thin autografts to hasten healing and improve "aesthetic disability." Despite her initial study being confined to small wounds, she commented that such treatment provided excellent graft take with decreased hospital inpatient pain and little need for follow up reconstructive procedures (Janzekovic 1972).

This programme was also adopted by burns surgeons in Boston. Burke et al showed that early excision and grafting of full thickness burns gave far better results than conservative treatment. Initial doubt however existed in the treatment of deep dermal burns. Nevertheless a study at the University of Washington showed that early excision and grafting of intermediate depth burns was superior to spontaneous healing, decreasing hospital stay and cost and the need for secondary reconstruction. These patients returned to work twice as fast as the non-operative group.

Early tangential excision and immediate grafting is now the international standard for burns care. This appropriate treatment is comparatively straight forward in burns of less than 30% total body surface area. With larger burns the availability of donor sites becomes a serious issue. Historically patients have had serial operations to re-use re-epithelialised donor sites. This prolonged reconstruction delays patient care and incurs greater donor site morbidity. It has been the desire of all burns surgeons to provide an effective means of immediate burns reconstruction without incurring increased burns morbidity.

1.3 Measures to reconstruct the burn wound

1.3.1 Autologous skin

The best material for wound coverage would be full thickness skin (Corps 1969) but the total area available is limited by closure of the donor site. The current "Gold Standard" is

still the split thickness skin graft, despite it's poor aesthetic and sometimes functional outcome. Problems with donor sites include fluid loss, infection, slow healing and scars. Hypertrophic and keloid scars both at the recipient and donor site provide morbidity with an inevitable chronic treatment that unfortunately is more common in the young. Meshing to a ratio of 1:1.5 may improve "take" by promoting drainage without adversely affecting the cosmetic appearance (Tanner 1964), which is unsatisfactory when a wider mesh is used. In patients with large burns, expansion is essential and several techniques have been described to achieve this (Nanchahal 1989) but the widely meshed graft (Tanner 1969) remains the most popular. Donor sites for split-thickness skin grafts may be very limited in patients with extensive burns and thus numerous alternatives to autologous skin have been developed.

1.3.2 Allogeneic skin

With concerns as to donor site morbidity and the limited availability of autologous tissue the reconstructive surgeon has looked elsewhere for limitless means of skin cover.

Meshing autologous split thickness skin to a ratio of 1:6 or greater leads to a decrease in the healing rate, which may be overcome by using less widely meshed viable allograft overlay (Alexander 1981) or similar techniques. Alternatively, autografts may be inserted into porcine xenograft (Ding 1983). Allogenic skin has been thought to provide an alternative means of wound cover but, except for one case reported by Takiuchi (Takiuchi 1982), allografts have not survived in the long term. Even when the dermal component of the allograft appeared to survive, the presence of allogeneic cells could not be demonstrated using a Y chromosome probe (Phipps and Clarke 1991) and rejection of allogenic skin was shown to be antigen specific. Depletion of the Langerhans cell population in the graft by ultraviolet irradiation and glucocorticoid treatment may prolong survival of the graft.

Manipulation of the donor skin to attenuate its antigenicity may be insufficient to lead to allograft survival as ingress of host Langerhans cells may induce class II antigen expression by donor keratinocytes (Krueger 1984). Immunosupression of the recipient proved successful but problems with immunodeficiency prevented further development of this idea until selective T cell suppression with cyclosporine became available (Hewitt 1990). Low dose treatment with cyclosporine prolonged allograft survival in experimental

animals and in patients the drug was effective in preventing rejection during the period of administration (Frame 1989) although rejection may not be morphologically apparent when meshed autografts are overlain with allograft. Cyclosporine inhibits DNA synthesis by donor keratinocytes but does not appear to affect the graft adversely to any significant degree (Kanitakis 1990)

Allogeneic skin may be frozen and banked in a manner that preserves its viability, ensuring availability as necessary. However, demand outstrips supply by a factor of 5-7 fold in the USA (May, Jr et al. 1984) and there is a real risk of transmission of infection, including HIV (Clarke 1987). These problems have stimulated the search for other forms of wound cover.

1.3.3 Synthetic skin substitutes

The many synthetic skin substitutes available work through different mechanisms and are best suited to different situations (Jones, Currie et al. 2002).

It has been shown that moist conditions assist the healing of partial thickness wounds and wound exudates promote keratinocyte proliferation (Madden 1986). This has encouraged the use of semi-permeable membranes and hydrocolloids as short term dressings. The characteristics of bilaminates with a porous, adherent lower layer and a less permeable outer component make them more suitable for the longer term coverage of full thickness skin defects. Growth factors such as epidermal growth factor may be incorporated into collagen based dressings (Stompro 1990) and impregnation of xenografts with uterine angiogenic factor has enhanced the production of granulation tissue (Burgos 1989). Skin substitutes only provide temporary coverage until skin grafts become available. The ideal remains immediate, permanent wound closure.

1.3.3.1 Cultured Autologous Keratinocyte Grafts

The potential for using epidermal cell suspensions consisting of a mixture of cell types to graft large full thickness skin defects has long been recognised but disaggregated cells do not achieve significant expansions. The description of a technique to achieve clonal growth of cells using lethally irradiated fibroblasts (Rheinwald and Green 1975) together with the use of growth factor and cholera toxin (Green, Kehinde et al. 1979) set the stage

for up to 10,000 fold expansion in keratinocyte numbers to permit clinical use. Culture without feeder cells fails to provide such expansion.

Sheets of cultured autologous keratinocytes (figure 1.6) were first used on patients with burns by O'Connor et al (O'Connor, Mulliken et al. 1981). Several other groups subsequently reported success in patients with burns (Hefton 1983). Cultured keratinocytes have also been used to graft leg ulcers (Leigh 1987) defects after excision of giant pigmented naevi (Gallico 1989), patients with epidermis bullosa and scalp necrosis.

The rate of take judged clinically in patients with leg ulcers is variable, often being in the region of 30% (Leigh 1987). Except for patients with severe concomitant medical problems (Meuli and Raghunath 1997), the technique is of little advantage, apart from avoiding donor site morbidity, which may be reduced by techniques such as applying a widely meshed graft on the donor area, and involves an expensive form of treatment for a common condition. Little benefit has been obtained in patients with epidermolysis bullosa (Gallico 1989). Eight children with giant hairy naevi treated with cultured autologous keratinocytes achieved a clinical "take" rate of 20-90%, compared to 67-92% for meshed skin graft (Gallico 1989). Furthermore some of the areas grafted with cultured autografts blistered spontaneously, leaving open wounds which necessitated split skin grating, and in other patients the areas grafted with keratinocytes alone contracted to 30% indicative of hypertrophic scarring compared to split thickness grafts which persisted at 95% of their original areas. Other workers have noted a contracture of cultured keratinocyte autografts of the order of 50% (Clugston 1991). The contracture observed in wounds may be limited by the presence of a dermal component. Elsewhere, cultured keratinocytes have been reported to lead to the same contracture as meshed split thickness skin grafts (Teepe 1990).

The problem of spontaneous blistering has also been described in patients with burns grafted with keratinocytes (Woodley 1988), this being especially prominent in areas with extensive dermal loss. Instability of the epithelium may be related to immaturity of the dermo-epidermal junction. Biopsies taken from two adults and two children grafted with cultured autologous keratinocytes onto fascia revealed the presence of many of the antigenic components of the basement membrane zone by day 10 with a discontinuous lamina densa, although there was a marked reduction or complete absence of collagen

bundles extending from the neodermis into fascia up to 4 ½ months later (Woodley 1988) and poor reconstitution of the elastic fibre network (Woodley 1990). Furthermore, suction blistering time was reduced. Separation occurring below the lamina densa in contrast to cleavage at the level of the lamina lucida in normal skin was observed in response to minimal trauma 11 weeks after grafting with autologous keratinocytes in an 11 year old patient with 98% burns. This occurred despite the presence of anchoring fibrils, although their attachment to dermal collagen was thought abnormal. In another study of 4 patients a lamina densa first became identifiable at 6 weeks and became continuous by 5 months (Aihara 1989). In contrast, in 21 paediatric patients grafted with autologous second passage keratinocytes onto beds of muscle fascia prepared for about 3 weeks with allograft, xenograft or Biobrane, components of the dermo-epidermal junction were identifiable within 1 week, and by one month the lamina densa was complete (Compton 1989). The number of anchoring fibrils approached that of normal skin within 1-2 years, and the collagen matured, with elastic fibres matching those of normal skin in 5 years. Biopsies at comparable times showed a relatively immature dermo-epidermal junction and dermal component in the intersticies of meshed split thickness skin grafts. However, when keratinocytes were grafted onto granulating wounds, some of which contained dermal elements, a lamina densa and anchoring fibrils were identifiable within 5 days (Faure 1987). It is difficult to reconcile these disparate results, but the nature of the graft bed may be of significance and the presence of a living dermal component is essential for the formation of anchoring fibrils, at least *in-vitro* (Briggaman, Dalldorf et al. 1971). It is interesting that the presence of rete ridges appeared to be related to the site of origin of the cultured keratinocytes. The patterns of keratinisation became normal after 1 year.

The recipient bed also influences keratinocyte graft "take" in patients with burns. Muscle fascia and early granulation tissue were superior to chronic granulating wounds (De Luca 1989), with mean rates of "take" of 47% and 15% respectively (Teepe 1990). In contrast to split skin, cultured keratinocytes fail to "take" on fat (Teepe 1990). A dermal bed led to much improved take (Madden 1986) and full thickness defects prepared with cadaver allograft resulted in a "take" of up to 90%, when some of the allogenic dermis was retained (Nave 1992).

In the presence of bacterial contamination cultured keratinocyte grafts fail (De Luca 1989; Teepe 1990), whilst meshed split skin grafts may succeed even in the presence of Staphylococcus Aureus and Pseudomonas Aeringenosa (De Luca 1989). Many bacterial

species commonly found on burns patients and not usually associated with failure of meshed split skin grafts cause cell detachment and cytotoxicity to cultured keratinocytes *in-vitro*. In the presence of clinical infection, an average take of 40% was achieved, in contrast to 77% in non-infected patients (Odessey 1992). Microbial colonisation may be reduced by excision of the burn wound within 10 days of the injury and by the application of cadaver allograft, with consequent improved "take" of cultured keratinocyte grafts(Odessey 1992). The use of topical and/or systemic antibiotics has also been recommended (Clugston 1991).

The take of cultured keratinocytes was reported to be inversely related to the age of the patient in one study (De Luca 1989) but a multi centre analysis of 104 patients found no relationship between graft take and age, sex, race, percentage area of burn or extent of full thickness injury (Odessey 1992). However the anatomical site influences "take," the posterior surfaces of the trunk and thighs being most difficult (Haith, Patton et al. 1992).

1.3.3.2 Cultured Allogeneic Keratinocyte Grafts

It takes at least 3 weeks to grow sufficient numbers of keratinocytes for grafting from a $1cm^2$ biopsy (Green, Kehinde et al. 1979). This delay in the availability of cultured autologous keratinocytes could conceivably be overcome by using allogeneic cells (Hefton 1983). And unlike allogeneic skin, the use of cultured keratinocytes should not be associated with the transmission of infectious agents such as HIV. The most important transplantation antigens in man are HLA DR, which are expressed by Langerhans cells but normally by keratinocytes. After culture for 7 days epidermal cells fail to express HLA DR antigens and do not stimulate allogeneic lymphocytes (Morhenn 1982) although HLA-DR expression can be induced in cultured keratinocytes by mononuclear inflammatory cells by the addition of gamma interferon. The expression HLA class I antigens by cultured keratinocytes is also attenuated (Nanchahal 1989). Cryo-preservation permits the application of allografts for long term storage and use at centres distant from the laboratory (De Luca 1989). Frozen cultured allogeneic keratinocyte sheets have been shown to be as effective as fresh allografts in accelerating wound healing (Eldad 1987). In the rat, keratinocyte allografts undergo acute rejection, which may be delayed by Cyclosporine. Similarly, despite the lack of clinical or histological evidence of acute rejection, cultured sex-mismatched keratinocytes grafted on to burn wounds and deep dermal beds following tattoo excision of chronic ulcers (Phillips 1990) survived for less

than a week as assessed by a Y chromosome probe (De Luca 1989). However, allogeneic keratinocytes applied to split skin graft donor sites were shown to survive for at least six weeks as determined by blood group antigen expression (Thivolet 1986), a finding confirmed using HLA class I antigens and more recently repeated using blood group antigens. The donor keratinocytes were replaced progressively rather than being rejected en masse. Cultured keratinocytes continue to express surface blood group antigens *in-vitro* but the objection has been raised that surface antigen expression may be altered during wound healing and their detection may be influenced by the use of organic solvents during the processing of the tissue for microscopy (Dabelsteen and Mackenzie 1976).
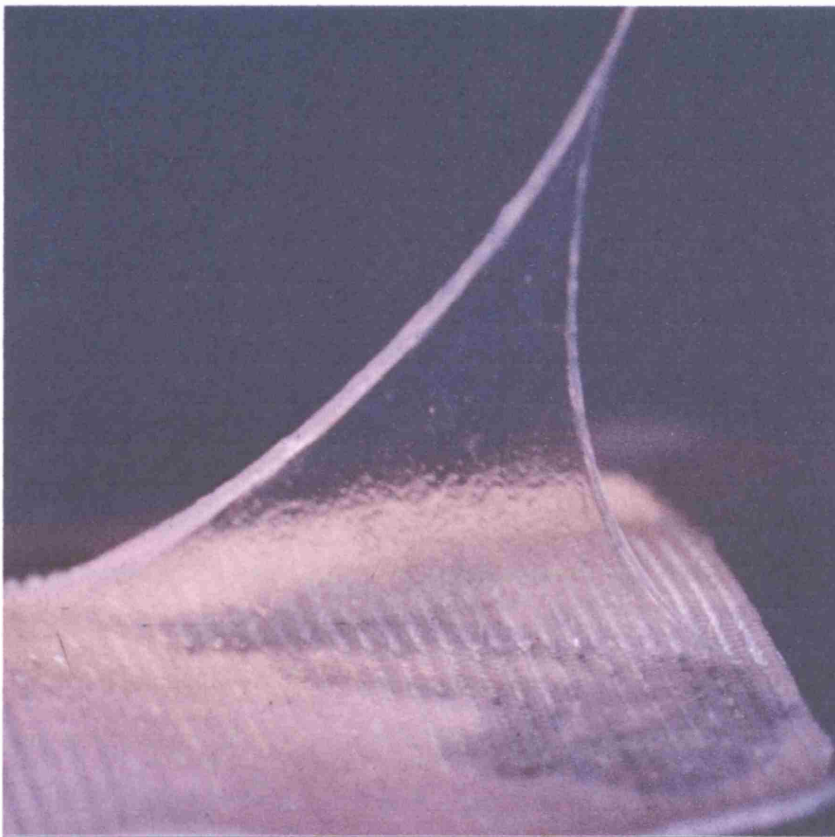
Figure 1.6 Cultured keratinocytes being removed from their transport medium

However Zhao was able to demonstrate the survival of some mismatched allogeneic keratinocytes for up to 92 days using a sensitive polymerase chain reaction technique to detect a Y chromosome specific DNA sequence. The survival of allogeneic keratinocytes appears to be related to the wound bed, deep dermal or full thickenss wounds resulting in early loss whilst the presence of the dermis in split skin graft donor sites leads to prolonged survival.

In chronic ulcers requiring multiple applications of sheets of cultured keratinocytes to achieve healing (Teepe 1990) they are clearly acting as biological dressings, perhaps by the release of growth factors. However on split skin donor sites, they show no improvement in the healing rate compared to a semi permeable membrane (Blight 1991). The problems associated with cultured keratinocytes alone have led to many burns units in the UK abandoning their use in favour of grafts with a dermal component.

1.3.3.3 Autologous keratinocytes on allogeneic dermal grafts

As an alternative technique to using allogeneic keratinocyte sheets, immediate cover may be achieved with cadaveric skin when the epidermis, representing the most antigenic component is removed and replaced with autologous keratinocytes. This was initially assessed in rats and then successfully applied to patients, in whom the roofs of 5mm suction blisters were placed on the dermal surface after the removal of the allogeneic epidermis at 3-5days (Heck 1985). Isolated autologous keratinocytes have been inoculated into cadaver allograft and in the two patients where the technique was successful, only 2 and 4 fold expansions of the autologous areas were achieved. A similar technique, but using sheets of cultured autologous keratinocytes to cover the dermal surface of allogeneic skin following removal of the epidermis by abrasion, achieved a 16 fold expansion (Cuono 1986). In a follow up of 2 patients' grafts by this technique, ultrastructural reconstitution of the dermo-epidermal junction commenced at 7 weeks and was complete by 13 weeks. The blistering seen in one of the patients resolved by this time and rete ridges were observed at 11 months. Encouraging results have also been reported from another centre (Nave 1992), two patients with 80-90% burns being able to return to occupations involving manual labour within 10 months of injury. The technique offers all the advantages of incorporation of a dermal component in the graft but retains the problems associated with the use of allogeneic dermis.

1.3.3.4 Acellular "Synthetic Dermal Equivalent"

The ideal alternative to cadaveric dermal allograft would be available in unlimited quantities, be easily stored and avoid the risk of transmission of infection. A bilayer " artificial skin" (Integra) consisting of a dermal component of collagen and a glycosaminoglycan (chondroitin-6-sulphate) overlaid with a silastic sheet meets some of

these criteria (Burke 1981) (Figure 1.7). The architecture of the dermal component is important, the pore size being a critical factor in determining the in-growth of host tissue. The type and proportion of glycosaminoglycan and cross linking with glutaraldehyde determine the physiochemical properties of the membrane (Yannas 1980), and determine degradation susceptibility. The silastic layer controls bacterial ingress, water evaporation and provides additional mechanical support.

Figure 1.7 An acellular Synthetic Dermal Equivalent (Integra) used to provide skin cover to a lower limb wound

In an initial study, 10 patients with extensive third degree burns had 15-60% of their total body surface area grafted with this material (Burke 1981). The silastic layer was removed after a few days and the denuded surface covered with thin split skin grafts. Problems encountered included haematoma collection under the "artificial skin," wrinkling with accumulation of a serous exudate and premature separation of the siliastic (figure 1.8). The incorporation of a slow release preparation of antibiotic showed a 38% decrease in infection rate by one group (Matsuda 1991). A multi centre randomised trial involving 106 patients found a median "take" rate of 80% compared to 95% for all control sites which included meshed autologous split skin. "Artificial skin" take was equivalent to that of cadaver allograft and the median value of the take of the thin split skin graft after removal of the silastic was 90%. The donor sites of the thin 0.15 mm split skin grafts healed quickly, there was less hypertrophic scarring associated with artificial skin compared to control sites and the cosmetic appearance was superior to meshed autograft. Histological follow up for up to 2 years on patients treated by this technique showed that

Figure 1.8 Infection, haematoma and serous collection with resultant poor graft take in a patient who underwent acellular synthetic dermal skin replacement following tumour excision

the dermal component was gradually remodelled over a period of 1 month, giving rise to a structure resembling papillary and reticular dermis, although no rete ridges were present.

1.3.3.5 Cellular "artificial skin"

The major disadvantage of artificial skin is the necessity for a second procedure to achieve epidermal cover, with all the attendant disadvantages of split skin donor sites. The obvious solution was the use of cultured keratinocytes, but initial attempts to cover the surface of the dermal component with these were unsuccessful as the matrix failed to support cell growth and they had a tendency to migrate down from the surface (Yannas 1984).

Autologous uncultured dermal and epidermal cell suspensions seeded at the dermal silastic interface by centrifugation led to less contraction of full thickness wounds on guinea pigs compared to acellular artificial skin (Yannas 1989). The grafts were rapidly vascularised with the large number of basophils possibly contributing to this process by the release of histamine. Within one year, a relatively normal dermal architecture had been restored, with the presence of rete ridges. Alternatively the surface of the dermal component of artificial skin without the silastic sheet has been covered *in-vitro* with cultured human keratinocytes (Boyce 1988).

Hemidesmosomes and extracellular matrix ultrastructurally resembling a basal lamina were apparent after 11 days in culture (Boyce 1988). The dermal component was populated by host fibroblasts after grafting. In order to avoid this delay a more organotypic cellular bilayer composite graft was prepared by inoculating cultured autologous fibroblasts into a porous surface and a non-porous collagen chondroitin 6 sulphate membrane laminated to the opposite surface was covered with cultured autologous keratinocytes, achieving a 10-20 fold expansion (Hansbrough 1989). These grafts were applied to 4 patients who had undergone excision of full thickness burns and preparation of the wound bed with allograft. There was graft "take" rate of 70%, failure being associated with bacterial colonisation. Within 9 days of grafting there was evidence of reconstitution of the basement membrane zone with the presence of anchoring fibrils, a much shorter time scale when compared to grafts of cultured keratinocyte sheets alone. This technique shows great promise for resurfacing skin defects, although the use of autologous keratinocytes does not permit immediate coverage.

1.3.3.6 Neovascularisation and tissue engineering

The largest restraint on advances in tissue engineering is arguably the current inability to vascularise large tissue engineered constructs mentioned above. In attempt to overcome this, various models of revascularisation have been developed, and different approaches to construction have been tried. These have been classified on the origin of their vascular supply namely;

1. Intrinsic blood supply – the engineered tissue may be transferred in a fully fabricated state and anastomosed to a blood supply and venous drainage at the defect site

2. An avascular tissue – this form of tissue engineered construct requires no circulation and is therefore avascular, as in the case of engineered cartilage (Sims 1998). A second method uses either an extracorporeal circulation of the patients' blood through a container containing xenografted endocrine cells or individual cells within microspheres. These immuno-isolation devices permit the infusion of oxygen and nutrients and the expulsion of waste products and endocrine factors, but do not allow the blood to come into contact with the cells themselves, which would instigate an immune reaction (Maki 1991).

3. Extrinsic blood supply - this is currently the most frequent method of neovascularisation in tissue engineering. The structure is grafted onto a rich vascular bed, vascular in-growth occurs from surrounding tissues. Re-vascularisation occurs via an endogenous response to the surgical grafting which creates an inflammatory wound healing response, this combined with the implant hypoxia encourages endogenous expression of angiogenic growth factors. Vascularisation can be enhanced by incorporating exogenous angiogenic growth factors in to the implant (Grzowski 2003).

   The tissue engineered construct may thus be designed to allow a rapid de-novo vascular in-growth and acts as a porous scaffold for the propagation of de-novo vessels. The angiogenic response may be encouraged by pre-seeding in-vitro with cells of a specific phenotype or post-seeding by external infusion (Wake 1995). Constructs need to be sufficiently thin to conventionally allow effective and rapid revascularisation and survival. Larger grafts give structural rigidity to prevent compression and destruction but pose a substantial angiogenic barrier. Angiogenic

agonists within such matrices however could allow the effective endothelial cell invasion, revascularisation and integration to promote take.

The potential of a bioengineered graft to promote angiogenesis is thus crucial in the process of graft take.

1.4 Angiogenesis

Angiogenesis is the formation of new blood vessels by a process of sprouting from pre-existing vessels. It occurs during development and post natal life and is required for the maintenance of functional and structural integrity of the organism post-natally.

The molecular basis of angiogenesis is most easily characterised by viewing the process as a step wise progression (figure 1.9). The initial vasodilation of existing vessels is accompanied by increases in permeability and degradation of surrounding matrix, which allows activated and proliferating endothelial cells to migrate and form lumens. The endothelial cells of these "sprouting" new vessels differentiate to accommodate local requirements, supported by a surrounding network of peri-endothelial cells (pericytes) and matrix, all of which mature through remodelling into a complex vascular network, ultimately lined by endothelial cells that have acquired critical survival factors, optimised to function under a variety of conditions. Angiogenesis is a robust cellular response which can arise from a variety of different stimuli.

1.4.1 Existing vessels dilate, vascular permeability increases and extracellular matrix is degraded

One mechanism of angiogenesis serves as an example. Largely in response to nitric oxide (NO), vasodilation is one of the earliest steps in angiogenesis perhaps due to high fluid sheer or hypoxia. VEGF, transcriptionally upregulated in part by NO, mediates an increase in vascular permeability, accomplished through redistribution of intercellular adhesion molecules, including platelet endothelial cell adhesion molecule (PECAM-1) and vascular endothelial (VE) cadherin and alterations in cell membrane structure via induction of a series of kinases (Eliceiri 1999; Gale 1999). Extravasation of plasma proteins follows, similarly induced by VEGF. Some of these create a temporary support structure through which activated endothelial cells subsequently migrate. Not surprising,

Blood capillaries form a dense network of microscopic vessels throughout the tissues, and are normally very stable structures.

After wounding, inflammatory factors signal the endothelial cells to become active and grow into the wounded tissue.

Angiogenesis starts with Endothelial Cell sprouting

Endothelial Cells then multiply and invade the tissue matrix

These cells establish adhesive contacts with each other, they form a string-like structure

Finally, the adhesive contacts become extensive and the cells remodel, forming a new capillary
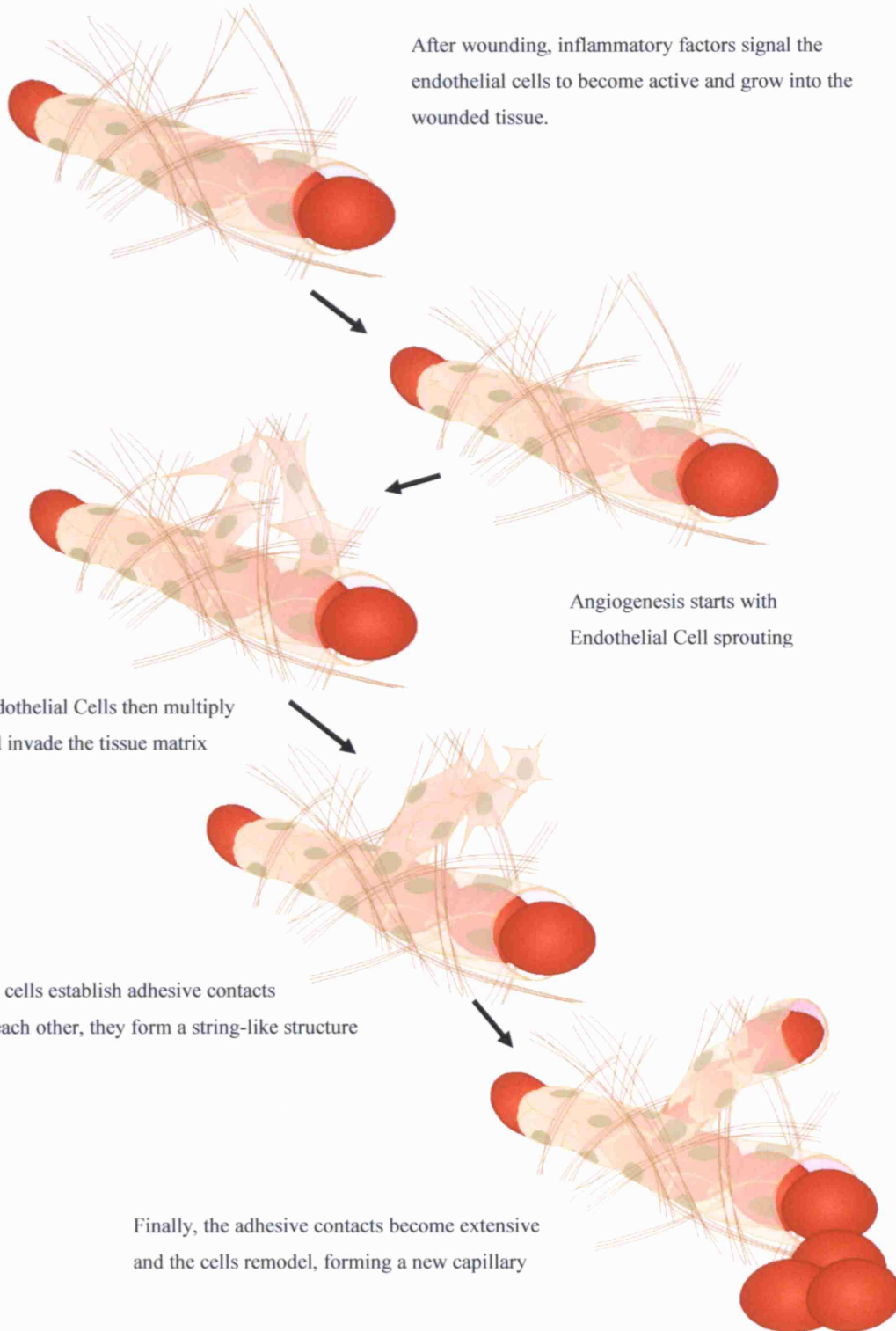
Figure 1.9 Angiogenesis. Cellular processes in the development of new blood vessels from the existing vasculature

excessive vascular permeability could result in pathological outcomes, such as intracranial hypertension or circulatory collapse. Consequently, permeability changes must be tightly regulated. Angiopoietin-1 (Ang-1), a ligand for the endothelial receptor Tie2, is a natural anti permeability factor that provides protection and a balance against excessive plasma leakage (Thurston 2000).

Endothelial sprouting is further enhanced by another member of the angiopoitin family, Ang2, an inhibitor of Tie2 signalling and a natural antagonist of Ang1, Ang2, appearing at angiogenic and vascular remodelling sites, is involved in stimulating smooth muscle cells and resulting in cell migration through the underlying matrix, thereby allowing endothelial cells to migrate as inter-endothelial cell contacts are reduced (Maisonpierre 1997; Gale 1999). Degradation of extracellular matrix involves an array of proteinases which not only provides "room" for the migrating endothelial cells, but also results in the liberation of growth factors, including bFGF, VEGF and insulin like growth factor (IGF-1), which can be sequestered within the matrix. The Matrix metalloproteinases are an important family of proteases implicated in angiogenesis and proliferation and over 20 are now known and implicated in angiogenesis and cell proliferation (Nelson 2000). In addition, they can expose or release cryptic adhesion sites, hidden in non-proteolysed matrix components (e.g. angiostatin, endostatin). As the name implies, MMPs play a central role in degrading extracellular membranes and basement membrane structures, allowing endothelial migration to occur. Natural inhibitors of MMPs include circulating protease inhibitors, such as tissue localised inhibitors of matrix metalloproteinases (TIMPS) (Brew 2000). It is at least partly through the secretion of MMP-2, MMP-3 and MMP-9 and suppression of TIMP-2 that Ang1 induces sprouting (Kim 2000). Similarly MMPs 3,7 and 9 have been shown to induce angiogenesis in neonatal bones and tumours (Vu 1998). MMPs do not uniformly enhance angiogenesis. Temporal and spatial factors likely dictate their function. MMPs 1 and 3 may also inhibit tumour angiogenesis by interfering with binding of MMP2 to integrins (Brooks 1998), while MMPs 7 and 9 generate angiostatin from circulating plasminogen, thereby inhibiting endothelial cell proliferation (Pozzi 2000). Interactions with other proteins also alter their roles in angiogenesis.Thrombospondin-1 (TSP-1) is believed to be anti-angiogenic by preventing activation of MMP-2 and MMP-9 (Bein 2000). Optimal angiogenic function of MMP-9 may require cell surface localisation with the hyaluronan receptor CD44 and TGFß (Yu 2000).

Other proteinases have also been implicated in matrix degradation enabling endothelial cell migration. Notably, urokinase type plasminogen activator (UPA, intrinsically involved in the plasmin system of fibrin degradation) is essential for revascularisation of myocardial infarcts (Heymans 1999) while antagonists of UPA or its interaction with the UPA have *in-vivo* anti-angiogenic therapeutic potential (Carmeliet 1998). A fine tuned balance between proteinases and their inhibitors is important to regulate angiogenesis. For example excessive plasmin proteolysis prevents pathological angiogenesis in inflammation (Bajou 1998).

1.4.2 Endothelial cell proliferation and migration

As the physical barriers are dissolved, migration pathways established, and cell to cell adhesions being "plastic," proliferating endothelial cells are free to migrate to distant sites. Interplay between the various forms of VEGF, angiopoietins, FGFs and their receptors are responsible for mediating, as major factors, these processes in embryonic, neonatal and pathological angiogenesis, although additional factors have also been implicated in the process. There may conceivably be considerable "redundancy" in these systems although several distinct functions for many of the components are emerging (Kerbel 2005). Although VEGF has profound effects throughout angiogenesis (Ferrara 2000), homologues of VEGF are more restricted. For example placental growth factor (PIGF) (Persico 1999) may function as a "decoy" attenuator in embryonic vascular development, but an essential amplifier of VEGF driven angiogenesis during pathological conditions. VEGF-B has been implicated in extracellular matrix degeneration via activation of plasminogen and is believed to regulate coronary artery function (Bellomo 2000). VEGF-C has been demonstrated to stimulate angiogenesis and lymphogenesis in the adult, although its endogenous role in pathology remains undefined (Veikkola 2000). Its receptor VEGFR-3 is highly expressed during embryonic development and is required for vascular remodelling and angiogenesis (Veikkola 2000). Ang – 1 via phosphorylation of Tie2, is chemotactic for endothelial cells, induces sprouting and stimulates the interaction between endothelial and peri-endothelial cells (Suri 1996). Ang-2 in concert with VEGF is also angiogenic, although in the absence of VEGF, Ang 2 may actually induce vessel regression (Maisonpierre 1997).

Fibroblast growth factors (FGFs) stimulate endothelial cell growth and recruit mesenchymal and/or inflammatory cells, producing many angiogenic factors (Carmeliet

2000). Platelet derived growth factor (PDGF) is angiogenic for microvasular sprouting endothelial cells and recruits pericytes and smooth muscle cells around nascent vessel sprouts (Lindahl 1998). From gene inactivation studies in mice, endothelial nitric oxide synthase (eNOS) was determined to have *in-vivo* angiogenic properties, inducing endothelial growth following denudation injury or hind limb ischaemia (Murohara 1998). Additional studies have demonstrated that VEGF stimulates eNOS and a release of NO. Members of the TGFβ superfamily, such as activin A are multifunctional cytokines that have been variably reported to both stimulate and inhibit endothelial growth in different models (Pepper 1997). TGFβ1 suppresses tumour angiogenesis, while TNFα modulates VEGF induced endothelial proliferation via interference with VEGFR-2 phosphorylation (Guo 2000). TNF also stimulates VEGFR2 expression in some models. More recently, several chemokines including monocyte chemotactic protein (MCP-1) have been demonstrated to induce endothelial growth (Belperio 2000). As the endothelial cells proliferate and migrate, directed in part by signalling through integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (Eliceiri 1999) PECAM-1 and Eph/epherin receptor ligand pairs (Huynh-Do 1999), they contact other endothelial cells. Endothelial cell junctions are established with adhesion molecules such as VE-cadherin and members of the connexin family (Knudsen 1998). Integrins may localise MMP to the endothelial cell surface and extracellular matrix thereby explaining why antagonists of integrins might be therapeutically effective anti-angiogenic agents.

Numerous additional molecules with angiogenic or anti-angiogenic effects following administration in various animal models have been identified. Those that are angiogenic are among others erythropoietin, insulin, IGF-1, neuropeptides, leptin, epidermal growth factor, hepatocyte growth factor, interleukins, monocyte activating peptides (Carmeliet and Jain 2000). Similarly several natural inhibitors of endothelial cell proliferation have been uncovered (e.g. TGFß, thrombospondin, endostatin, angiostatin).

1.4.3 Endothelial cells assemble, form cords and acquire lumens

As endothelial cells migrate into the extracellular matrix, they assemble into cell cords and subsequently acquire a lumen. There are several mechanisms described for lumen formation such as the fusion of cellular vesicles or a vasculogenic pattern of remodelling. Lumen diameter is tightly regulated by several factors. While $VEGF_{121}$ and $VEGF_{165}$ and their receptors increase lumen formation, $VEGF_{189}$ decreases lumen diameter. In

combination with VEGF, Ang-1 augments lumen diameter (Suri 1998). Multiple integrins are also involved in lumen formation and cylclic RGD compounds may abrogate their function (Bayless, Salazar R. et al. 2000). Finally there are also several endogenous inhibitors of lumen formation, including thrombospondin -1 (TSP-1) and tubedown (tbdn-1) (76).

1.4.4 Long term survival of vascular endothelium

A remodelling phase of angiogenesis has been observed in many systems. This involves firstly apoptosis and secondly a transition of activated cells to a stable quiescent phenotype. Once new vessels have been assembled, the endothelial cells become remarkably resistant to exogenous factors and are quiescent. Diminished endothelial survival or endothelial apoptosis is characterised by vascular regression in the embryo (Carmeliet 1999). Several factors identified that regulate endothelial apoptosis such as bFGF, and Tie$_1$ have been identified (Kockx and M.W. 2000) and these vary considerably according to the developmental time point, the specific site, function and type of vessel, in addition to surrounding physiological and pathological stimuli. Deprivation of nutrients following obstruction of vessels by spasm or thrombi, results in release of pro-apoptotic signals and effective suppression of anti-apoptotic stimuli, leading to endothelial programmed cell death and vessel regression. In premature babies, exposure to hyperoxia reduces VEGF levels and causes retinal vessel regression (Alon 1995).

The molecular mechanisms by which a quiescent, confluent endothelium is able to maintain its physiological function in various vascular beds for long periods of time is unclear. However some insights have been gained from *in-vivo* and *in-vitro* studies. The role of VEGF as a survival factor depends on its interaction with VEGFR-2, PI3Kinase, β-catenin and VE cadherin (Carmeliet 1999). Lack of the cytoplasmic domain of VE cadherin results in endothelial apoptosis via interruption of VEGF signalling, leading to diminished activation of the protein kinase Akt and lack of upregulation of the anti-apoptotic *bcl-2* gene (Carmeliet 1999). Other factors known to play a role in endothelial survival include angiopoetins via their cognate receptors Tie1 and Tie2, Ang 1 promotes, while Ang2 suppresses survival, particularly with respect to tumour vessel growth (Holash 1999). A mechanism through which Ang 1 promotes survival is by enhancing expression of the anti- apoptotic gene, survivin, via activation of Akt through Tie2 signalling (Papapetropoulos 2000).

Maintenance of vascular integrity in different vascular beds also requires haemodynamic shear forces, which not only provide appropriate metabolic requirements to target tissues, but regulate and reduce endothelial cell turnover and abrogate endothelial apoptosis caused by TNF-α (Dimmeler 1996). Molecular mechanisms implicated in mediating cell cycle arrest and survival of vascular endothelial cells include several factors involved in regulation of cell cycle and apoptosis, such as p53, p21, p16, p27, BAX and p42/44 mitogen activated protein kinase (Pages 2000). Other angiogenesis inhibitors also provide survival advantages to endothelium (e.g. prothrombin kringles-1 and -2, thrombospondin-2, antagonists of PECAM-1, interleukins 4 and 12 and cyclooxygenase 2 (COX) inhibitors).

1.4.5 Critical role of peri-endothlial cells and surrounding matrix

Although the endothelium has received the most attention in angiogenesis research, the surrounding pericytes cell layers and extracellular matrix are critical for ongoing structural and functional support of the vascular network. Pericytes and vascular smooth muscle cells stabilise nascent vessels by inhibiting endothelial cell proliferation and migration. Indeed, vessels regress more easily when not covered by smooth muscle cells in case angiogenic stimuli become more limiting (Lindahl 1999). Peri-endothelial cells are metabolically active, and express a variety of vasoactive peptides, growth factors and cytokines that impact on the overall function of the vasculature. The extracellular matrix is also critical for normal vessel growth and maintenance, by providing not only a "solid" scaffold through which new vessels may migrate, but to store and mobilise necessary growth factors, to mediate appropriate intercellular signals. Overall an understanding of the means by which peri-endothlial cells are recruited and migrate, differentiate to serve a local function, and finally interface with the extracellular matrix and endothelium, is essential to ultimately design angiogenic matrices.

Recruitment of peri-endothelial cells is mediated by an array of local factors. PDGF-BB is chemoattractant for smooth muscle cells (Lindahl 1998) and VEGF, possibly via release of PDGF or binding to VEGF receptors, also contributes. Interactions between the endothelial cells of nascent vessels and the peri-endothelial cells are stabilised by Ang-1 and Tie2 the latter of which also induces branching and remodelling (Lindahl 1998). There is strong clinical evidence of the importance of these receptor ligand systems in

vessel maturation. Dysfunction of Tie2 results in diminished smooth muscle cells and the vascular malformations in humans. Members of the TGFβ family, including TGFβ1 together with TGFβ receptor 2, endogolin and Smad 5, also work in concert to induce vessel maturation by stimulating smooth muscle cells differentiation and extracellular matrix deposition, while inhibiting endothelial proliferation and migration.

The extracellular matrix is critical for angiogenesis and should be viewed as a dynamic player in the process. In addition to providing a site for storage of growth factors and pro-enzymes, such as the MMPs, for release and activation, its components serve as binding sites and targets for endothelial and mesenchymal cell-derived integrins and growth factors. For example integrin $\alpha_v\beta_3$ that mediates attachment to denatured collagen and provisional ECM components such as fibronectin is essential for vessel survival and blood vessel maturation during angiogenesis and inhibition of this interaction results in apoptosis, vessel regression and interference of the angiogenic effects of VEGF and TGFβ (Brooks, Clark R.A. et al. 1994). Other matrix components include fibronectin, laminin, vitronectin, fibrin and hyaluronic acid each of which variably interacts with integrins and other growth factors, facilitating endothelial and peri-endothelial cell migration, tube formation and vascular network maturation.

1.5 Angiogenesis and synthetic skin

The thorough analysis of all the known components involved in angiogenesis for potential use in a second generation pro-angiogenic Synthetic Dermal Equivalent (SDE) is well beyond the scope of this thesis. However this thesis aims to evaluate the efficiency of a broad range of known angiogenic mediators on endothelial cell migration. Invasion, infiltration into scaffolds and cell migration were also considered to be the most important cell behaviours to focus on for improving on existing skin equivalents. Although no single model fully represents a wound environment, a practical strategy to compare the angiogenic effects of such a large array of factors would be to utilise an *in-vitro* assay of some essential part of the in-vivo angiogenesis response.

1.5.1 *In-vitro* models of angiogenesis

Two-dimensional models of endothelial cell migration/invasion which have been frequently used as convenient *in-vitro* assay systems, do not appropriately reproduce *in-situ* conditions. For instance, TGFβ was reported to inhibit endothelial cell proliferation

in two dimensional systems but not in a three dimensional system (Madri, Pratt B.M. et al. 1988). As a consequence, three dimensional systems have been frequently used; endothelial cells were grown within collagen or fibrin gels and stimulated by angiogenic factors to invade their supporting scaffold (Madri, Pratt B.M. et al. 1988). However the complexity of the growth response made it almost impossible to accurately quantify capillary formation in these systems (Williams 1993). A different type of three dimensional angiogenesis assay was proposed by Nicosia et al (Nicosia and Tuszynski 1994); whole tissue segments (rings) of the rat aorta, embedded in fibrin or collagen matrices, gave rise to capillary-like structures after some days of *in-vitro* culture. However it was not proved that these structures were composed of endothelial cells. This thesis uses two in vitro assays;

1. A proven reliable three dimensional *in vitro* system (the cytoball assay) that allows quantification of migratory response of cultured endothelial cells exposed *in-vitro* to various growth factors and components of the extracellular matrix. (Nehls and Drenckhahn 1995). This assay is used for the angiogenic analysis of; extracellular matrices (chapter 3), growth factors (chapter 4), components of fibrin synthesis/degradation (chapter 5) and Ultrasound (chapter 6).

2. A novel *in-vitro* assay of endothelial cell migration through Synthetic Dermis with or without the presence of varied Topical Negative Pressure (chapter 7).

A Synthetic Dermal Equivalent needs to provide reliable results if it is to have an effective therapeutic role in the treatment of large wounds. The ability of present SDEs to adhere and integrate with the wound (take) is compromised as they fail to allow endothelial cell ingress and angiogenesis. This problem has been selected for investigation *in-vitro* using a combination of two assays. The following observations have been made regarding SDEs;

1. Clinical take of current SDEs (Integra) is suboptimal, especially on large wounds, which are the most critical to treat. It is also relatively slow.

2. The observed failure to take is specific to the SDE as split thickness grafts are extremely reliable where they can be used (take about 95%).

3. Failure to take is associated with infection, seroma or haematoma but increasing evidence points to a failure of angiogenesis.

4. Angiogenesis can fail in the wound environment in spite of high levels of Angiogenic Growth Factors. The possibility that EC/ECM signals e.g. from Integrins may be relevant particularly to populating new tissue territory warrants consideration.

5. Pro-angiogenic SDEs could provide a novel second generation dermal replacement.

1.6 Hypotheses

In noting the above clinical problems and observations this thesis aims to improve SDE take by testing the following hypotheses;

1. SDEs fail to successfully take due to constituents which impair angiogenesis.

2. ECMs are classically considered to be permissive scaffolds to cellular motility and activity.

3. The central hypothesis of the thesis is that ECM components interact with endothelial cells to determine cellular migratory behaviour rather than acting as permissive scaffolds.

4. Angiogenic growth factors act synergistically with ECMs to determine cellular migratory ingress.

5. Structural properties of SDEs influence cellular ingress.

6. Wound healing adjuncts stimulate cellular motility within SDE matrices to promote angiogenesis.

1.7 Experimental Aims

1. To assess and compare the performance of extracellular matrices in allowing endothelial cell invasion and thus the potential revascularisation of dermal equivalents (chapter 3)

2. To assess the stimulus of growth factors in increasing endothelial cell invasion through extracellular matrices (in comparisons to ECMs) for potential use in a second generation Synthetic Dermal Equivalent (chapters 4 and 5)

3. To study the effects of ultrasound on endothelial cell invasion *in-vitro* as a potential therapeutic avenue for increasing SDE take (chapter 6).

4. To study the effects and compare regimes of TNP in increasing endothelial cell invasion into SDEs (chapter 7)

5. To study SDE matrix scaffold with or without TNP in providing an optimum environment for endothelial cell invasion (chapter 7)

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Tissue Culture/Cytoball assay

All cell cultures were carried out in sterile Class II laminar air-flow hoods (LaminAir), provided by Heraeus Instruments. Tissue culture was undertaken in Heraeus temperature controlled (37° C), humidified, CO_2 (5%) incubators. Centrifugation was performed in a Heraues Labofuge 4000. Microscope examination was achieved using an Olympus CK2 inverted phase contrast microscope. Cells were counted in a Fuchs Rosenthal haemocytometer (Marienfield). All refrigeration used Creda Cold store fridges for 4 and – 20 °C and New Brunswick Scientific freezers for –80 °C. All components were weighed on AND HR60 scales using 7ml and 100 ml square weigh boats (Fischer).

Endothelial cells were isolated and cultured from human placentae, these samples were initially sieved using a 50 Gauge mesh (Sigma). Resultant cells were further sieved following enzymatic break down using 90, 70 and 40μm meshes (Biomesh, Merck).

All culture medium was stored at 4°C and included: Dulbecco's phosphate buffered saline (PBS) for tissue culture and M199 with Earle's salts and 25mM Hepes. These were all obtained from Gibco LTD. The foetal calf serum was obtained from European Community cattle (Sigma) and the Platelet Depleted Plasma – derived serum (PDP) from First Link. All other tissue culture components are shown below,

Table 2.1 Manufacturing suppliers of components for tissue culture

Component	Company
UW Cold storage solution	Du pont pharmaceuticals Ltd
Gentamicin	Life technologies
Cold hank's balanced salt solution	Life technologies
Dispase	Sigma
Collagenase II A	Sigma
Dnase type I	Sigma
Anti-thrombomodulin antibody (QB/END40)	Serotec
Anti-mouse immunoglobulin-coated	Dynal
Human AB positive serum	First link
Penicillin	Gibco

Streptomycin	Gibco
Fungizone	Gibco
Ceftazidine	Glaxo Welcome
GlutaMax-I (glutaminyl-alanine)	Gibco
Heparin	Sigma
Trypsin	Gibco
Versene (1:5000)	Gibco
Thrombin	Sigma
Plasmin (human)	Calbiochem
Epsilon aminocaproic acid	Sigma
Aprotonin (10,000Kiu/ml)	Ferring Pharmaceuticals
Hirudin	Sigma
Paraformaldehyde	BDH laboratory supplies
Agarose gel (low melting point)	Gibco
10x M199	Sigma

Plastic ware including culture flasks, universal, filters and 24 well plates were supplied by
Griener Labortechnik. Extracellular matrix components were from a variety of sources
(table 2.2) as were the angiogenic/growth factors and receptor blockers (table 2.3).

Table 2.2 Sources of extracellular components

Collagen 1 (rat tail)	First Link UK LTD
Fibrinogen Fraction 1 (bovine)	Sigma
Thrombin (human)	Sigma
Vitronectin	Sigma
Fibronectin (bovine plasma)	Sigma
Laminin –A (human placenta)	Sigma
Elastin (bovine neck ligament)	Sigma
Hyaluronic acid (human umbilical cord)	Sigma
Dermatan Sulphate	Sigma
Chondroitin Sulphate A (bovine trachea)	Sigma
Heparan Sulphate sodium salt (bovine kidney)	Sigma

Growth factors, where appropriate, were reconstituted in water from a lyophilised state. These solutions were diluted and aliquoted using PBS (Mg 2+ + Ca 2+) and stored at the appropriate temperature (-20, -80C). When needed, the aliquoted samples were thawed and diluted to the required concentrations using minimal media.

Collagen coated Cytodex spheres (Cytoballs) were obtained from Pharmacia Biotech.

Table 2.3 Suppliers and properties of growth factors used

Growth Factor	Source	ED50
Acidic FGF (ECGS)	First link	10 ng/ml
Basic FGF	First Link	0.5 ng/mL
PDGF BB	First Link	1 – 3 ng/ml
VEGF-A$_{(165)}$	First Link	10-100 ng/mL
TGFβ	First Link	< 0.2 ng/ml
TNF-α	First Link	0.01-0.1 ng/ml
TPA	Sigma	1.3 +/- 5.5ng/ml
IL1ß	First Link	5 ng/ml
IL8-(77aa)	First Link	1-5 ng/ml

2.1.2 Gel exclusion Chromatography

Protein chromatographic fractionation was performed using a Hi prep ™ 26/60 Sephacryl ™S-100 high resolution gel filtration column (volume 320 mls) using a Biorad LC1 System. Associated tubing and connectors were of 1mm diameter (Biorad) and all chromatographic fractions collected in polystyrene universal containers. The system was set up with a five ml sample tube (Bio-Rad). Fractions were detected by changes in UV absorbance (A 280) and eluent was also monitored for conductance. This data was recorded by a dual pen chart recorder (Bio-Rad 1327) at 12 cm/hr.

The buffer was made using 0.01M Hepes (BDH), 0.5M Sodium chloride (BDH), 5M NaOH (Sigma) to reach a pH of 7.4 and distilled water (Walker and Nesheim 1999) and stored in 2L glass containers (Fisher Brand). The markers; Cytochrome C, Carbonic Anhydrase, Albumin, Alcohol dehydrogenase, β amylase, Blue Dextran (Sigma) were dissolved in Dulbecco's PBS pH 7.4 and used to calibrate the column (chapter 2.3). Protein elutes were concentrated using Vivaspin VS 2002 (25 ml, 10kDa cut off)

ultrafiltration tubes and the concentrations determined using a Bradford protein assay (Biorad).

2.1.3 SDS PAGE gels

The following solutions were used;

1) Acrylamide/Bis Acrylamide premixed 37.5:1 (Sigma)
2) 1M Tris-HCl (Sigma), pH adjusted to 6.8 with 10M HCl (BDH)
3) 1.5M Tris-HCl (Sigma), pH adjustment to 8.8 with 10M HCl
4) 10% (w/v) Sodium Dodecyl Sulphate (SDS, BDH)
5) 10% Ammonium Persulphate (AmPs, BDH)
6) TEMED (Invitrogen)
7) Coomasie stain - made with 0.25% Brilliant Blue R (Sigma) with 10% acetic acid (BDH) and 45% methanol (BDH)
8) Destain - 10% acetic acid and 45% methanol
9) Running buffer – 0.025 M Tris, 0.192 M Glycine (BDH), 0.1% SDS
10) Precision Plus Protein ™ standards (all blue, Bio-Rad)
11) 8M Urea (BDH), 5%SDS, Tris HCL pH6.9, non boil, non reducing buffer

A Biorad minigel electrophoresis system and power pack were used; a R100 Rotatest shaker (Luckham) for staining/destaining and 3 MM grade Chromatography paper (Whatman®) following destaining for drying and storage. This was achieved by synchronous vacuum (Laboport KNF Neuberger pump) application to heat pad (GP40/50 gel dryer, Gibco) for 1 hour. All gels were protected by lamination (Ibico laminator and pouches)

2.1.4 Ultrasound

Ultrasonic energy was delivered to cytoball cell cultures in gels on 24 well plates using a Bosch Sonomed 4 with SK4350 Bosch handset (diameter 5cm[2]. Contact between endothelial cell cultures and ultrasound probe was maintained using Ultrasonic™, a generic ultrasound transmission gel.

Figure 2.1 Bosch Sonomed 4 used for ultrasound generation

2.1.5 Topical Negative Pressure

Polycarbonate chambers with interposing Polytetrafuoroethylene washers (depth of 2mm and 1mm) were made by the bioengineering department at Mount Vernon Hospital. The chambers comprise two interlocking sections, an upper "A" and a lower "B" section illustrated below.

Figure 2.2 Section A of the TNP *in-vitro* pumps (dimensions in mm)
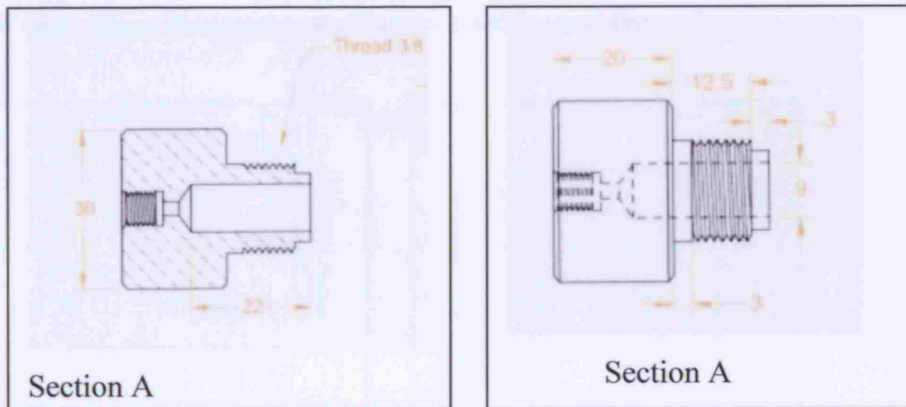
Section A

Section A

Each section has a Luer lock port for attachment to external pressure tubing or 20 ml syringes (Teruma).

Figure 2.3 Section B of the in-vitro TNP pumps
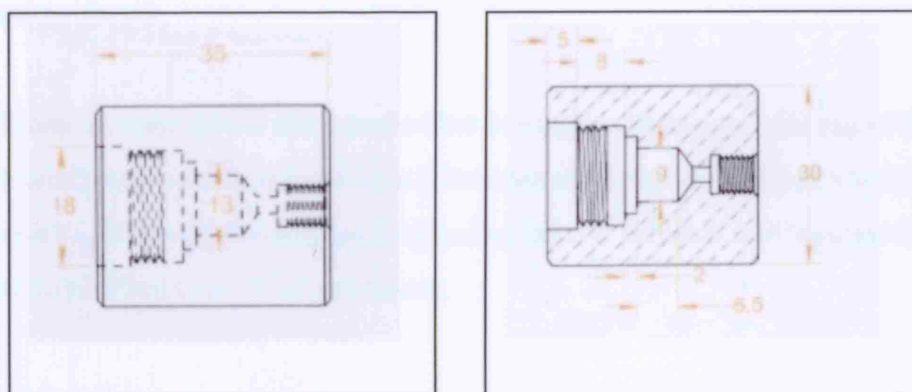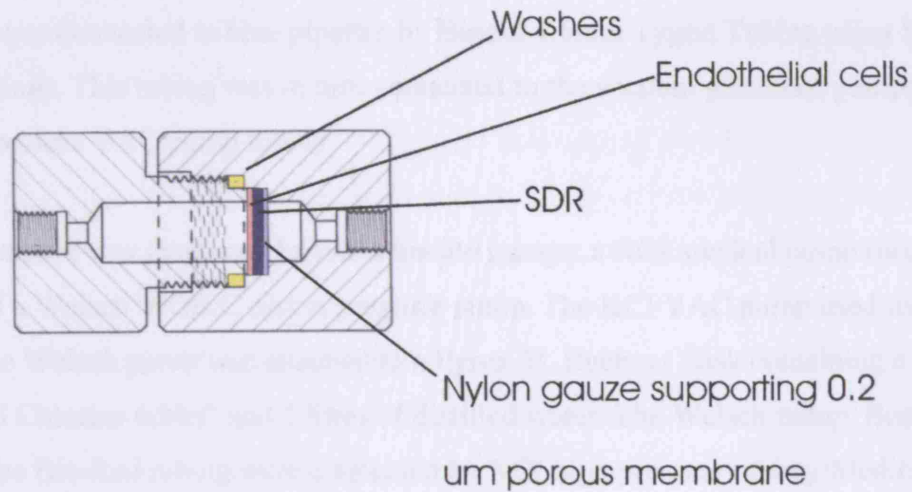
Figure 2.4 Section A screws clockwise into B.

The investigated Synthetic Dermal Equivalent is supported between the two sections by a Nylon gauze (Rexaloy GMX – 500 – V, Fischer) and a 0.2μm pore polycarbonate GTTP membrane (Millipore Isopore™). These were cut to size by a nine gauge Rexaloy Cork Borer. All materials were cleaned using Virkon® (Antel International) and sterilised in a Boxer 400/150V Autoclave at 121 °C for 15 minutes at 15 Psi prior to use. Parafilm "M"® (American National Can ™) was used to seal the units.
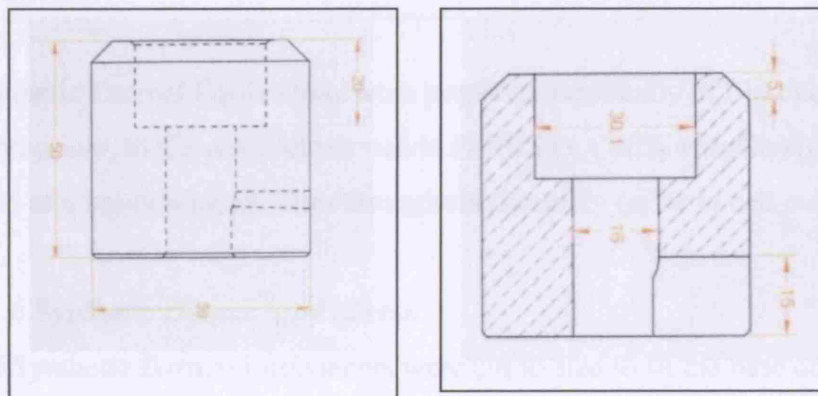
Figure 2.5 Each unit was supported by a base made from black Delrin, dimensions and features as shown.

All chambers, once sterile were assembled in aseptic conditions. All negative pressure experiments were conducted in Class II laminar air-flow hoods (LaminAir), Heraeus Instruments. Vacuum was applied to the chambers in Heraeus temperature controlled (37° C) and humidified CO_2 (5%) incubators.

Chambers were connected to blue pipettes by Biorad 1.6mm Tygon Tubing using Biorad pressure fittings. This tubing was in turn connected to the vacuum generator pumps by KCI high pressure VAC pump tubing.

Negative pressure was generated by two separate pumps: a KCI medical pump (non Trac™) and a Welsch WOB.C piston pressure pump. The KCI VAC pump used its own canister. The Welsch pump was attached to a Pyrex 5L Buchner flask containing a single +1 "Hospital Chlorine tablet" and 2 litres of distilled water. The Welsch pump, Buchner flasks and the Bio-Rad tubing were connected by KCI high pressure tubing. Medicell mediclips were used to clamp the tubing when appropriate.

The following Synthetic Dermal Equivalents were studied;

Table 2.4 The dermal equivalents studied for their ability to allow endothelial cell ingress with/out TNP

Synthetic Dermal Equivalent	Company
Integra®	Integra Life Sciences Cooperation
Permacol™	Tissue science laboratories
Alloderm®	Life cell
Xenoderm®	Medical biomaterial Products GmbH

Synthetic Dermal Equivalents were prepared aseptically in Bio one Petri dishes. Where appropriate, SDEs were rehydrated in D-PBS (+ $CaCl_2$ +$MgCl_2$) (Gibco). 5% serum was used as a nourishing medium throughout the study (refer to cell culture).

2.1.6 Synthetic Dermal Equivalents

All Synthetic Dermal Equivalents were cut to size to fit the base on the *in-vitro* TNP pump using the Rexaloy borer. With the exception of Xenoderm the SDEs also required sharp cutting excision.

2.1.6.1 Alloderm

The Alloderm was aseptically removed from the all packaging. The Alloderm was completely submerged and rehydrated in 200 mls of PBS in a Petrie dish. At this stage the backing material was removed and discarded using sterile forceps. After 30 minutes the

SDE was transferred to a further Petrie dish and the process repeated. An even thickness confirmed full rehydration.

Alloderm has a distinct upper and lower surface. When applied to a wound bed the dermal surface must be placed against the wound bed with the basement membrane surface facing up. In our experiment the endothelial cells had to be placed on the dermal surface.

Table 2.5 One can determine the orientation of Alloderm based on physical characteristics (the author's preferred method of assessment of orientation was texture and colour).

Basement Membrane side (to be lying down in the *In-vitro* TNP pumps)	Dermal side (face up in TNP pumps, in contact with endothelial cells)
Dull	Shiny
Rough	Smooth
Buff-coloured	White

2.1.6.2 Integra

Under sterile conditions the outer package was peeled open and the inner foil pouch removed. The pouch was inverted a few times and holding the pouch vertically (notched end up) the top of the foil pouch was cut, any Isopropyl alcohol was drained off. Using a sterile technique the Integra was removed from the foil pouch and into a sterile dish for washing. At this stage the SDE was checked for any defects or contaminants. The Integra was subsequently rinsed in 2 litres of normal saline solution for at least one minute and the process repeated. The silicone black threads on the epidermal side confirmed Integra orientation. This side was placed down in the TNP pump with the collagen exposed dermal side facing upwards. The silicone was scored through and through with a blade, without contact with the underlying collagen dermis, prior to insertion into the TNP pumps.

2.1.6.3 Permacol

Permacol required no preparation or rehydration prior to use and thus only necessitated sharp cutting excision prior to chamber placement.

2.1.6.4 Xenoderm

Once removed from the packaging, Xenoderm was rehydrated in 200mls of PBS in a Petrie dish for 30 minutes. Following rehydration the circular sections of Xenoderm were harvested as described above.

2.1.7 Image analysis

Images were taken using a DC200 camera on an inverse Nikon Eclipse TS100 light microscope or a Zeiss Axioskop inverse phase light microscope at 10 x magnification. Images were recorded using a Leica IM50 image manager with image size 1280 x 1024. Cellular invasion was calculated using Sigma Scan Pro image measurement software™. Data was statistically analysed using Jandel Sigmastat 2® statistical software

2.2 Methods

2.2.1 Collection of placentas and processing of placental tissue

Endothelial cells were cultured from human placentae obtained with full patient consent and the relevant approval of the local ethics committee. Placentas were harvested from St Mary's Hospital NHS trust Paddington, London and Manchester. Seven placentas were processed within 30 minutes of delivery following spontaneous labour or elective caesarean section from control pregnancies (35-42 weeks gestation) with no antenatal complications apart from a preterm labour. Ten samples (1-2cm^3) of placental tissue were excised from the basal surface of different cotyledons and thoroughly washed in PBS containing 100μg/ml Gentamicin. Six pieces of placental tissue were retained for endothelial cell isolation and four pieces of tissue were fixed in 4% paraformaldehyde in PBS, pH 7.4 for immunohistochemistry. Placental tissue was routinely stored overnight in a cold storage solution (Viaspin, Du-Pont pharmaceuticals Ltd) prior to the endothelial cell isolation procedure.

2.2.2 Isolation of HPMEC

HPMEC were cultured from purified capillary fragments (Leach L, Bhasin Y et al. 1994). Placental tissue was washed in cold hank's balanced salt solution containing 100μg/ml Gentamicin (HBSS/G) and finely dissected to release chorionic villi which were sieved

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(50 Gauge mesh) to yield intermediate and terminal villous fragments. Following washing twice by centrifugation in HBSS/G (200xg for 5 min), villi underwent enzymatic disaggregation with 0.25% trypsin in HBSS/G for 10 min at 37°C, which was terminated by washing twice with HBSS/G plus 1% foetal bovine serum (HBSS/FBS) Tissue was further disaggregated with collagenase IIA (500U/ml) and Dispase (25 U/ml) in Medium199 with 5% FBS, for up to 2 hours at 37°C with gentle agitation. Following two washes in HBSS/G, tissue was treated with trypsin (2.5mg/ml) and DNAase type I (0.1mg/ml) in HBSS/G for 15 minutes at 37°C. This step was terminated by washing in cold HBSS/FBS, followed by titration through 19 followed by 21 gauge needles and sequential sieving of the tissue fragments through 90, 70 and 40μm meshes and recovery by centrifugation of the filtrate. Positive immunoselection of capillary fragments was accomplished as follows: incubation for 30 minutes at 4°C with anti-thrombomodulin antibody (QB/END40) bound to anti-mouse immunoglobulin-coated P450 Dynabeads at a ratio of 0.75 bead/cell, and five cycles of magnetic separation and washing in HBSS/FBS. The resulting suspension was concentrated by centrifugation and re-suspended in 0.5 ml of complete medium (M199 with Earle's salts, bicarbonate and HEPES supplemented with 20% human AB positive serum, hypoxanthine/ Thymidine mixture, 2nM Glutamax , 100 U/ml penicillin, 100μg/ml streptomycin and 2μg/ml fungizone, 100μg/ml endothelial cell growth supplement (ECGS) and 100μg/ml heparin). Aliquots (40μl) of this suspension were added to culture plates pre-coated with 1 ml gelatin and pre-equilibrated with 1ml of complete medium. Cultures were maintained at 37°C in 95% air/ 5% CO_2 with three weekly changes of media. Individual colonies of putative endothelial cells in low-density cultures, as evidenced by cobblestone morphology, were picked and subcultured. The serum concentration in the medium was reduced to 5% when confluence was attained. The phenotype of HPMEC cultures in this study (passage 3-12) was characterised using a panel of markers (Dye 2001). HPMEC are found to express ACE, eNOS, PAL-E, A10-33/1 and the blood group H antigen and ability to proliferate in response to VEGF.

2.2.3 Cell culture

All culture flasks were pre-coated with 1% gelatine (bovine skin, Sigma), incubated at 37°C for 24 hours and the excess gelatine aspirated. Flasks were sealed and stored until needed.

Endothelial cells were cultured *in-vitro* in 25 or 75 ml culture flasks in M199 25mM with Earle's salts supplemented by 5% PDP, 2% foetal calf serum, Penicillin, Streptomycin, Ceftazidine and Glutamax 1 at 37°C with 95% air, 5% CO_2. All media was exchanged three times per week. Cells were split using 0.25% trypsin in versene (1ml to T25 or 3ml to T75 flasks). Cells were manually agitated from the flask base and culturing media immediately pipetted into each flask (6 mls for the 25 ml flask, 10 mls for the 75 ml flask) to neutralise further trypsin activity. Cells were pipetted into sterile conical containers and centrifuged for 7 minutes at 1000rpm. Subsequently cells were resuspended in 1 ml of M199 culture media or, if for the Cytoball assay, a minimal media (MM, culturing media with decreased serum - 0.1% Bovine Serum Albumin (BSA), 0.5% Foetal Calf Serum (FCS). Cell counts were performed under ten times magnification using an haemocytometer by diluting the cell suspension 1:10 with trypan blue 0.4% (sigma). Cells were passaged using split ratios between 1:3 to 1:7.

2.2.4 Cytodex bead assay

Cytodex beads™ were swollen and hydrated in PBS without Ca^{2+}/Mg^{2+} (50 ml/g) for 3 hours at room temperature. The supernatant was decanted and beads washed once for 5 minutes in Ca^{2+}/Mg^2 free PBS (50 ml/g) and autoclaved at 115 °C for 15 minutes at 15 Psi. The suspension was allowed to settle and stored at 4°C thereafter for extended periods until use.

Twenty-four well plates were pre-coated with 100μl of sterile 1% (LMP) agarose gel (Sigma) per well. 100 μl of cytodex sphere suspension (approximately 250 spheres), 750,000 endothelial HPMEC cells and 500 μl of MM, were added per well. The cells and beads were incubated at 37°C for 24 hrs to establish confluent cell growth (figure 2.6).

Endothelial cell migration assays were established by suspending confluent beads in ECM gels. Approximately thirty beads were measured per condition or variable. This quantity of beads was established by adding 3 drops of cytoball suspension to 10 mls of extracellular matrix mixture. The solution was then gently pipetted to mix evenly and transferred to three 24 well plates. All experiments used triplicate cell lines. Any further components needed for ECM gelling (ie thrombin) were placed at the well bases prior to ECM addition. Once gelled (30 minutes), equal volumes of media were added to the matrix.

The media and gel were incubated at 37 °C with 5% CO_2 for 96 hrs, all media was exchanged at 48 hrs and the gels fixed (1ml of 4% Paraformaldehyde) at 96 hrs. Images of migration were taken following fixation. An average cell invasion distance from each bead was calculated using Image Measurement Software™. For each cytoball, in each experimental condition, ten migrating cells chosen radially, and measured for invasive distance from the beads circumference (figure 2.6 lower). This was performed in an ordered repeatable fashion, starting at the superior aspect of the bead, moving 36° anticlockwise for each subsequent cell. For each experimental condition a mean migratory distance and standard error of the mean was established from thirty cytoballs (i.e. three hundred cells). From the thirty beads, for each condition, a mean migratory distance and standard error of the mean was established. This process was repeated with at least three cell lines for each variable.

2.2.5 Statistical Analysis

Differences in migration from minimal media were determined using a one-way ANOVA and Tukey post hoc test (Sigma Stat 2 statistical package software). All migration values were normalised to migration in 0.75 mg/ml collagen with minimal media angiogenic stimulation (minimal media migration thus given a value of 1). This was termed the invasion coefficient, and allowed statistical assessment and comparison between all three cell lines. Thus for each experiment the mean invasion in pixels for each condition in each cell line was converted to microns, using a formula derived from using a stage graticule;-

Distance of migration in microns = distance in pixels x 0.658761528

2.2.6 Extracellular matrix synthesis

All extracellular matrices, excluding collagen were sterile filtered (200μm) prior to *in-vitro* assay.

2.2.6.1 Collagen gels

Type 1 Collagen (rat tail collagen type 1 in 0.6% acetic acid) was prepared in sterile conditions as follows; 10x M199 at 1/10th the volume of collagen was added to $NaHCO_3$

at 1/100th the volume of collagen, then collagen. The solution then neutralised with
NaOH. Further dilutions were made with minimal medium.
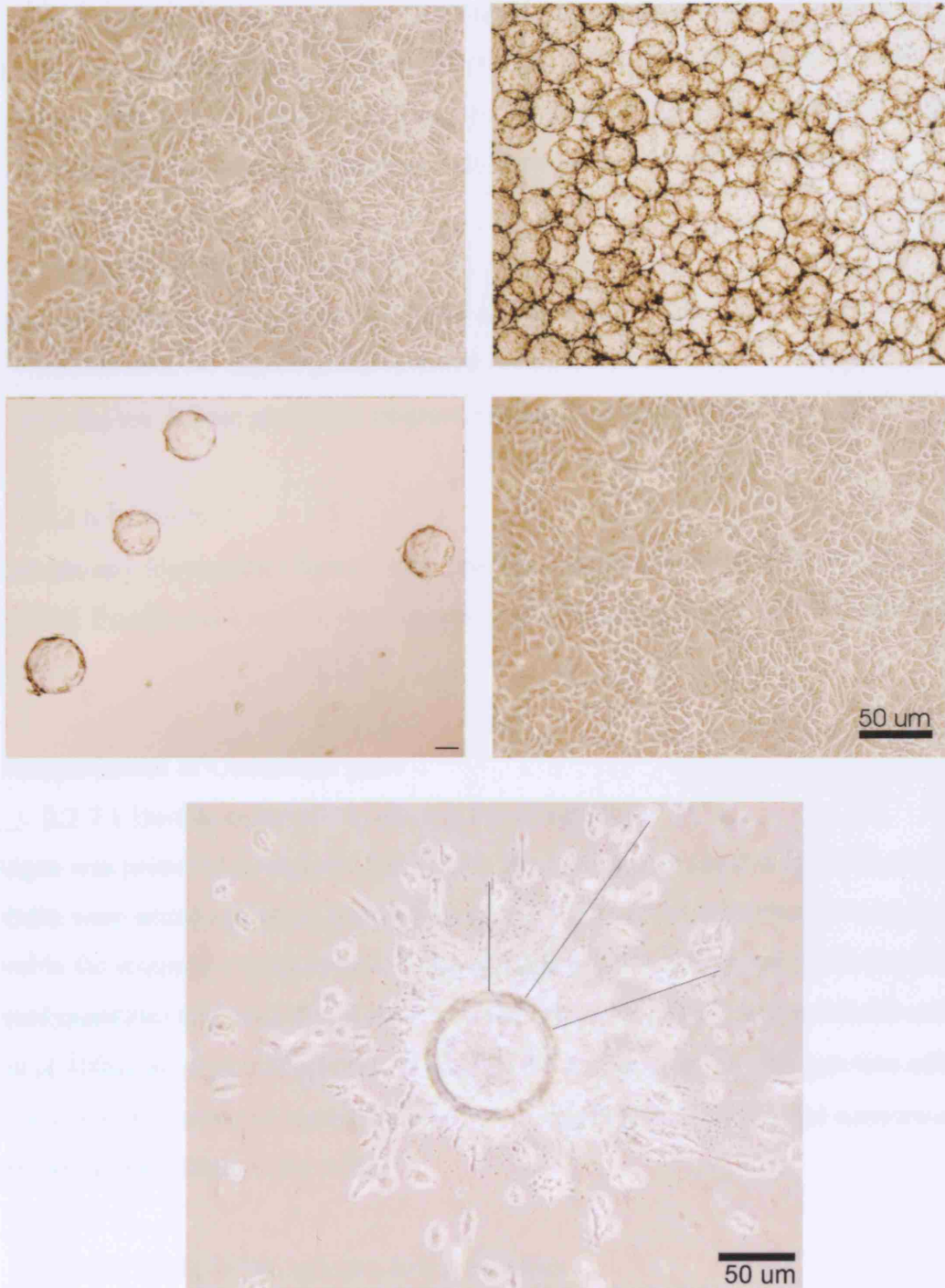
Figure 2.6

The regular cobblestone morphology of confluent endothelial cells (upper left).
Endothelial cells cultured on cytodex sheres (upper right) are suspended in collagen
(middle left), following 96 hrs of co culture with media, the cells have invaded the matrix
(middle right), this invasion is quantified by image analysis (three out of the ten cells to
be measured marked, bottom). Scale bars = 50 μm.

2.2.6.2 Fibrin gels

Fibrinogen, bovine fraction 1 type IV fibrinogen, was dissolved in minimal media at 37°C, sterile filtered, and stored on ice. Thrombin was dissolved in M199 with 0.1% BSA at 10U/ml and stored in aliquots at 80°C. Pilot studies had showed that 0.8 units of thrombin was sufficient to polymerise 1 mg of fibrinogen. Thrombin was thus pre-plated to all 24 well plates at the appropriate amount prior to the addition of fibrin.

2.2.6.3 Glycosaminoglycans

Chondroitin sulphate and dermatan sulphate and hyaluronan were prepared at concentrations of 0.1-1 mg/ml using minimal media. Heparan sulphate was prepared at 0.01 – 0.1 mg/ml. Where necessary preparation was aided by manual stirring.

2.2.6.4 Others

Vitronectin and fibronectin (Sigma) were prepared at concentrations of 0.01 - 20μg/ml using MM. Elastin and Laminin were prepared at 0.1-1mg/ml and 0.1875- 0.75 mg/ml respectively.

2.2.7 Manufacture of Composite gels

2.2.7.1 Double component collagen based matrices

Collagen was prepared as described at double the required concentration (1.5mg/ml), the cytoballs were added and the solution kept on ice. Other ECM components were prepared at double the required concentration. Both the Collagen and additional ECM were added in equal quantities and once thoroughly blended they were mixed with cytoballs and plated at 400μl per well and allowed to set. For the control gel, the collagen was added to equal quantities of minimal media, mixed thoroughly to give a 0.75mg/ml concentration and subsequently added to the wells.

2.2.7.2 Triple ECM collagen based matrices

Collagen was prepared at twice the concentration as above and mixed with cytoballs. The further two ECM components were prepared at four times the required concentration and added to each other, vigorously mixed, then this double matrix solution added in equal quantities to the collagen/cytoball gel and then plated.

All wells containing double or triple ECM matrices with fibrinogen were pre-plated with thrombin as above.

2.2.8 Extraction of soluble components from collagen

Collagen 0.75 mg/ml was prepared as described and incubated for 30 minutes at 37°C. Once gelled the collagen was spun at 4000rpm for 1 hr, the supernatant aspirated and the insoluble collagen precipitate discarded.

2.2.9 Addition of angiogenic factors

Angiogenic factors were taken from stocks and diluted to the necessary concentrations using Minimal Media. These solutions were added to gelled collagen matrices (section 2.2.6.1). All angiogenic factors were replaced with the minimal media after forty eight hours of incubation. This was achieved by gently aspirating each well with the plate held at ten degrees. The replaced media was added drop-wise to avoid disruption of gel structure.

2.3 Gel exclusion chromatography

All buffers were degassed by 0.2μm vacuum filtration prior to use. Gel exclusion chromatography was performed using a pharmacia S100 column. Prior to initial use, the pump was purged to remove all air within the system. The column was mounted vertically and connected with care to avoid any air entry. The column was initially equilibrated with one half-column volume of filtered distilled water at a flow rate of 1.3ml/min with a subsequent addition of two column volumes of buffer at 2.6 ml/min. The column efficiency was tested and then calibrated using gel filtration molecular weight markers;

Table 2.6 Molecular markers used to calibrate the chromatography column

Marker	Molecular weight, kDa
Cytochrome C from horse heart	12.4
Carbonic Anhydrase from bovine Erythrocytes	29
Albumin, Bovine serum	66
Alcohol Dehdrogenase from yeast	150
β-Amylase from sweet potato	200
Blue Dextran	2000

An equal quantity (1mg) of each marker was added to the column and the volume for mid peak elution noted (V_R). These values were each divided by the volume of elution for Blue Dextran (V_E). The V_R/V_E values were plotted against the log of the molecular weight of each marker as shown overleaf.

The separating buffer used was 0.5 M NaCl, 50mM Hepes, pH 7.4 (Walker and Nesheim 1999). When rested the column was stored in 20% Ethanol.

Gel exclusion calibration 18-21/5/04	KDa	ln(KDa)	chart speed 12cm/hr flow 1.3 ml/min 6.5ml/cm cm	vol	0.2cm/min 5min/cm vr/ve
Blue dextran	2000	7.600902	14.6	94.9	1
Amylase	200	5.298317	17.4	113.1	1.1917808
ADH	150	5.010635	19	123.5	1.3013699
BSA	66	4.189655	21.3	138.45	1.4589041
Carbonic anhydras	29	3.367296	27.4	178.1	1.8767123
CytC	12	2.484907	31.2	202.8	2.1369863
col vol				320	3.3719705

Regression Output:

Constant	10.40467
Std Err of Y Est	0.706707
R Squared	0.873582
No. of Observations	6
Degrees of Freedom	4

X Coefficient(s)	-0.0405198
Std Err of Coef.	0.00770709

Calibration of Sephacryl S200

18-21/5/04
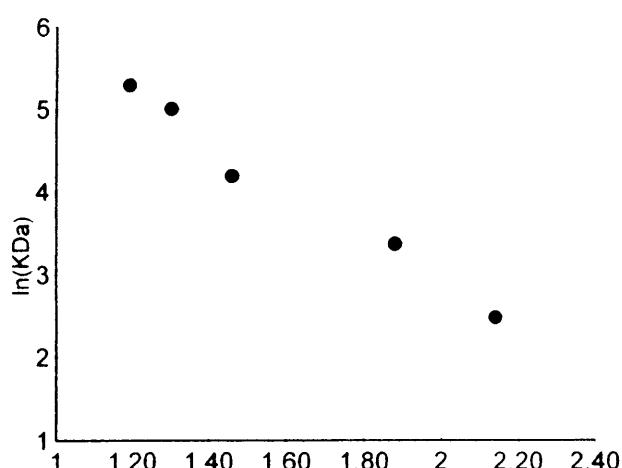
Figure 2.7 The linear distribution of molecular weight markers used for chromatographic column calibration.

2.3.1 Sample analysis

Prior to sample analysis the column was equilabrated with a one column volume of running buffer. Samples were sterile filtered and injected into the 5 ml injection loop whilst isolated from the running buffer by a distribution valve. The valve was switched to load the sample and a mark made simultaneously on the chart at time zero. With initial pilot studies, fractions were collected every 2 mls throughout the recording and those corresponding to changes in the absorbance retained and pooled. Subsequent runs with similar samples produced identical elution profiles and thus fractions were collected corresponding to specific portions of the profile. The points at which fractions were collected were marked on the chart.

Fractions were stored at 4°C and centrifuged at 4000 rpm using Vivaspin ultrafiltration devices to obtain a 1ml concentrate and remove any material below 10,000kDa.

SDS PAGE

The chromatographic fractions from FDPs were analysed by SDS-PAGE. Prior to electrophoresis all samples were mixed in a 1:2 ratio to the urea based non-reducing buffer (2.1.2).

8% gels were used to analyse the molecular weights of gel exclusion chromatography elutes. The proportion of each component is shown in table 2.7.

Table 2.7 Volumes (ml) of components used for SDS PAGE

Component	15ml, 8% Separating gel component volume	3 ml Stacking gel component volume
Distilled water	7.0	2.1
Acrylamide/Bis Acrylamide (37.5:1)	4.0	0.5
1M Tris-HCl pH6.8	0	0.38
1.5M Tris-HCl pH 8	33.8	0
10% SDS	0.15	0.03
10% Ammonium Persulphate	0.15	0.03
TEMED	0.009	0.003

Glass plates separated by 0.75 mm spacers at the lateral edges were clamped into a casting rig with a rubber seal at the base. The separating gel was pipetted between the two glass plates to 2/3 the height and allowed to set. The surface tension of the gel was decreased with water saturated tert-butanol. Once set the tert-butanol was drained off and a comb was inserted to form sample wells and the stacking gel was cast. The glass plate/gel assembly was transferred to an electrophoresis tank and clamped in a central holder with a blank glass spacer or further gel opposite, thus creating a peripheral area and central reservoir for running buffer within the electrophoresis tank. These areas were filled with running buffer to a level higher than the stacking gel to establish electrical connection through the gel. The 10 well spacer comb was removed from the gel allowing running buffer to fill the wells. Markers/samples were loaded into the wells by displacement with a fine gel loading pipette tip. Markers were loaded to the left and samples to the right.

Gels were run at 100V until the samples had fully entered the stacking gel, then following 60 minutes of electrophoresis at 200 volts the gels were transferred to a square Petrie dish containing Coomasie stain and oscillated for 1 hour. The stain was poured off and destain solution was exchanged until the protein markers became visible and the gel colourless. For preservation gels were transferred to paper and placed in a vacuum gel drier at 60°C for 1 hour and were then laminated.

2.5 Bio-Rad protein Assay

Protein concentrations of samples were estimated using a Bradford assay (biorad) measuring absorbance at 595 nm (Cam spec M330 spectrophotometer).

Table 2.8 The concentrations of BSA to determine protein concentrations

Vol of BSA (0.4mg/ml)	Vol PBS	Conc BSA (μg/ml)
0	50	0
3.125	46.875	1.25
6.25	43.75	2.5
12.5	37.5	5
25	25.0	10
50	0	20

Control protein solutions of 0.4mg/ml Bovine Seum Albumin in PBS were PBS. Initial stock concentrations of 0.4mg/ml BSA were further diluted in PBS in triplicate tubes as per table 2.8 to give the concentrations in the right hand column.

To each tube of BSA above, 375µl NaOH was added and the vortex mixed. Then, 375µl HCl and 200µl BioRad protein assay dye reagent were added with a further vortex mix.

Protein concentrations were similarly measured, typically in 10µl of sample which were mixed with 395µl of NaOH, 395µl of HCl and 200µl of BioRad dye reagent. Each sample was assayed in triplicate. The sample concentrations were determined from a graph of the BSA standards.

2.6 TNP chambers

2.6.1 Pump Assembly

The KCI "VAC pump" was assembled using the matched canister and reinforced cable. The locking end of the cable was removed and a 1 ml pipette tip attached.

The Welsch Pump was attached to a 5 litre Buchner flask with reinforced high pressure hose. The top of the jar was sealed with a pre-bored 7.5 cm diameter rubber stopper, sealed with a 10ml pipette, which in turn was connected to reinforced KCI pressure tubing. The KCI tubing had the locking end removed and a 1 ml pipette tip attached.

2.6.2 *In-vitro* TNP pump assembly

TNP pumps were disinfected between use in Virkon for 24 hours prior to manual cleaning and air-drying.

The 8mm Nylon mesh circles (chapter 2.1.5) were placed in the base of section A and the 0.2µm filter membrane was placed on top and held secure by a 2mm deep washer screwed close by the upper section.

The whole assembly and a 1 mm washer was autoclaved at 121 °C. Once sterilised and cooled the chamber assembly was unscrewed and the washer removed. The SDE under investigation, once cut to size, was placed onto the membrane and secured in place by

two washers (1mm and 2mm). The upper section was then screwed into the base section. The basal port (port B, figures 2.3, 2.4) was sealed using 1 cm^2 parafilm$^®$.

2.6.3 Endothelial cells

Endothelial cell were harvested from confluent cultures (N7/7, N6C2, N5C5) (figure 2.6, upper right). 5 x 10^5 cells in 1 ml of medium were added to the upper surface of each SDE within the chamber using a 1mm syringe and 21gauge needle through the Luer port. Cells were left for one hour prior drop by drop addition of 1 ml of minimal media (refer to Cytoball method) using an identical technique. The upper port was sealed with Parafilm$^®$ and the chambers, containing air, media, cells and Synthetic Dermal Equivalent were incubated at 37 C in 5% CO2 for 24 hours.

At 24 hours all six chambers were removed form the incubator and transferred to a sterile environment. The lower parafilm was removed and the vacuum tube attached using the Luer lock system. The opposite end on the hose was attached to reinforced pressure tubing from either the KCI vacuum apparatus or the Welsch pump via a yellow and blue pipette tip as connectors.

5 chambers were connected in parallel to the Welsch pump, one to the KCI pump. A 10 ml syringe barrel was inserted into the upper Luer lock port of each chamber. The reservoir was filled with tissue culture media (refer to cell culture) but an air space was kept within the vacuum pump itself (figure 2.8).

The syringe barrels were covered with Parafilm$^®$ to maintain sterility. The 6 chambers were labelled;
1) no TNP
2) 4 hours
3) continuous
4) Intermittent

Figure 2.8 The *in-vitro* TNP pump fully assembled with upper inverted media containing syringe and lower suction tubing.

Following pump connection, seals were tested under water using negative pressure at 125 mmHg. Negative pressure was applied via the connection tubing continuously to chambers 2 – 3 from the Welsch pump and intermittently to chamber 4 from the KCI pump (5 minutes on, 2 minutes off, 125 mmHg). The tubing to chamber 1 was clamped at all times. Tubes 2 was clamped after the appropriate time interval (1hour, 2 hours and 4 hours respectively). These clamps, to all but chamber 1, were released after 24 hours and the process repeated for a further 24 hours. Following 48 hours of Topical Negative

Pressure the vacuum was stopped, all chambers were unscrewed and the Synthetic Dermal Equivalents with adhered membrane (maintained for orientation) were placed in labelled 10 ml beakers containing 10% buffered formalin.

2.6.4 Migration analysis

Digital photographs were taken of 5 histological sections of each SDE for each condition using a Leica DC200 digital camera, Zeiss Axioskop microscope (10X magnification) and Sigma Scan Imaging package.

For each histological section the SDE thickness was calculated (B) by measuring the width of the SDE in 5 places (figure 2.9). Cellular invasion distance (A) and total depth of SDE was measured at these sites. The % invasion distance was calculated as;

$$\frac{A}{B} \quad X \quad 100$$

And the mean value per slide was recorded. 5 slides were taken of each condition and thus the average invasion for each Synthetic Dermal Equivalent with each mode of Negative Pressure Therapy established.
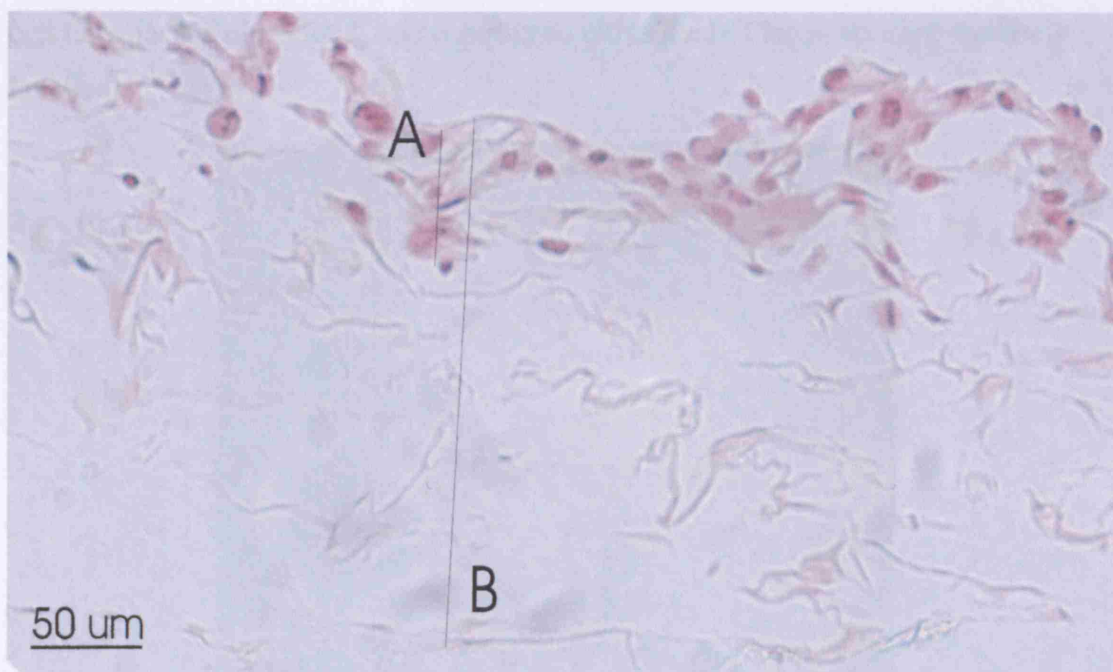
Figure 2.9 Following incubation of the SDEs with endothelial cells and media the SDEs were fixed and sections were taken for histology. Following staining, cellular migration was measured as a fraction of the depth of the matrix (A/B).

2.7 Histology

All histopathology was undertaken by Mrs Elizabeth Clayton at the RAFT institute. Specimens for sectioning were placed in 10% buffered formalin and fixed for twenty hours. Specimens were bisected and embedded on edge and further processed overnight in a Tissue Tek® VIP tissue processor. Paraffin samples were then cut into 4μm sections using a Reichel Jung Microtome taken on Snow Coat Extra slides (Surgipath St Neots Cambs), dewaxed and rehydrated using Xylene and graded concentrations of industrial methylated spirit. Samples were dried at 60°C for one hour. They were subsequently washed under running tap water prior to staining with Haematoxylin and Eosin. Samples were then dehydrated through graded concentrations of methylated spirit and permanently mounted using DPX. All histological solvents were from Genta Medical (York, England) other than DPX (RA Lamb London).

2.8 Ultrasound and angiogenic endothelial cell invasion

Cytoballs were prepared as described above (para 2.2.4) (cell lines N7/7, N6C2, N5C5) and plated in 24 well plates with 0.4ml Collagen 0.75mg/ml. Once gelled, equal quantities of Minimal Media were added. Eight wells were used per plate, two plates per cell line, one for ultrasound, one control (no ultrasound). Plates were appropriately labelled.

Figure 2.10 The application of Ultrasound to the cytoball experiment plate using a coupling gel.

Cytoballs within the collagen gels were incubated at 37°C at 5% CO_2 for 24 hours. Following this, plates for ultrasound treatment of each cell line underwent 15 minutes of 3.0 MHz pulsed ultrasound (2ms on 8 msec off) at 0.2, 0.8 and 3 W/cm^2 (figure 2.10). Hands were gloved to prevent ultrasound transmission to the investigator and the US probe moved at 30 rpm to avoid standing wave formation (moving applicator technique) (Coleman 1987). A coupling gel was used between the probe and the plate base to minimise any air steel interface impedance. This process was repeated every 24 hours. The cytoballs were fixed at 96 hrs in 4% paraformaldehyde (1ml per well).

Chapter 3

The investigation and comparison of effects of extracellular matrices on endothelial cell migration

3.1 Introduction

This chapter aims to evaluate potential components for inclusion in a second-generation pro-angiogenic Synthetic Dermal Equivalent (SDE) by means of an endothelial cell migration assay.

The Extracellular Matrix (ECM) is a vital component of all tissues and organs whose synthesis and degradation is dynamically regulated. The ECM is a complex mixture of structural and functional proteins, glycoproteins and proteoglycans all arranged in a unique tissue specific three-dimensional scaffold for tissue organ morphogenesis, maintenance and reconstruction. The many functions it serves at the molecular level include the provision of structural support and tensile strength of tissue, adhesive attachment sites for cells and as a reservoir for signalling factors such as growth factors and secondary regulatory factors such as heparin. Together these modulate such diverse host processes as cell migration, cell proliferation/morphogenesis, inflammation, immune responsiveness, wound healing and angiogenesis.

Dermal forms of ECM reside subjacent to structures that are rich in epithelial cells. These forms of ECM tend to be well vascularised, and comprise primarily type 1 collagen and site-specific glycosaminoglycans and a wide variety of growth factors including bFGF, VEGF and EGF (Goldman 2004). In contrast, the ECM of the basement membrane that resides immediately beneath the endothelial cells of blood vessels is comprised of distinctly different collections of proteins including laminin (Miyazaki 2006), collagen type IV and entactin (Charonis, Sideraki et al. 2005). Until the mid 1960s the ECM was assumed to be a passive support rather than an active determinant of cell behaviour. However with the discovery that the ECM plays a role in the conversion of myoblasts to myotubes (Hauschka and Konigsberg 1966) and that structural proteins such as collagen and glycosaminoglycans are important in salivary gland morphogenesis (Wessells 1968) it became obvious that the ECM is much more than a passive bystander in the events of tissue and organ development and in the host response to injury. The discovery that cytokines, growth factors and other bioactive proteins are contained within the ECM characterised it as a virtual information highway between cells. All ECMs share the common features of providing structural support and serving as a reservoir of growth factors and cytokines. The ECMs present these factors efficiently to resident cell surface receptors, protect the growth factors from degradation and modulate their synthesis

(Bonewald 1999). In this manner the ECM affects local concentrations and biologic activity of growth factors and cytokines and makes the ECM an ideal scaffold for tissue repair and reconstruction. The concept of "dynamic reciprocity" between the ECM and intracellular cytoskeletal and nuclear elements has become widely accepted (Ingber 1991). Attempts to translate such phenomena to therapeutic uses of ECM as scaffolds for tissue engineering application have recently been attempted.

Scaffolds for tissue reconstruction and replacement ought to have both appropriate structural and functional properties. However the distinction between structural and functional proteins is becoming increasingly blurred. Sequences within proteins originally thought to have purely structural properties have been identified and found to have significant and potent modulating effects upon cell behaviour. For example the RGD sequence that promotes adhesion of numerous cell types was first identified in the fibronectin molecule (Yamada 1984), a molecule originally described for its structural properties. This sequence occurs in several other scaffolding proteins such as Vitronectin, Collagens, Fibrinogen and Von Willebrand factor. Several other peptides have since been identified in "dual function" proteins including laminin, entactin, fibrinogen, types I and IV collagen and vitronectin (Humphries, Mould et al. 1991). Many other cryptic domains or motifs have been recently discovered among them the anti-angiogenic factors endostatin and alpha angiostatin (Davis, McConkey et al. 2003). If one considers the ECM to be a degradable bio-scaffold for implantation, both the structural and functional components are transient due to the rapid rate of degradation of ECM scaffolds *in-vivo* (Badylak, Kropp et al. 1998).

3.1.1 Principal components of the ECM

Collagens are the most abundant class of protein within the ECM. More than 20 distinct types of collagen have been identified. The primary structural collagen in mammalian tissues is type I collagen. The amino acid sequence of collagens has been highly conserved. For this reason allogeneic and xenogeneic sources of type I collagen have been long recognised as a useful scaffold for tissue repair with low antigenic potential. Hence bovine type I collagen is a widely used biologic scaffold for therapeutic applications due to its abundant source and its history of successful use.

The ECM is a rich source of numerous types of collagen and the relative concentrations and orientation of these collagens to each other provide specific environments for cell growth both *in-vitro* and *in-vivo*. Collagen types other than type I exist in various specific ECM (e.g. tendon, ligament, facia and mesentery). These alternative collagen types each provide distinct mechanical and physical properties to the ECM and contribute to the utility of the intact ECM (as opposed to isolated components of the ECM) as a scaffold for tissue repair. Type IV collagen is present within the basement membrane of all vascular structures and is an important ligand for endothelial cells. Type VII collagen is an important component of the anchoring fibrils of keratinocytes to the underlying basement membrane of the epidermis. Type VI collagen functions as a connector of functional proteins such as type I collagen, helping to provide a gel like consistency to the ECM. This diversity of collagens within a single tissue type is partially responsible for the distinctive biologic activity of natural ECM scaffolds and exemplifies the difficulty in recreating such a composite *in-vitro*.

The family of collagen proteins plays a dominant role in maintaining the integrity of various tissues and also has a number of other important functions. Collagens form polymeric assemblies; fibrils, networks, filaments which serve as anchors for cellular adhesion via integrins: collagens are thus widely used as the core scaffold for SDEs (Cairns, deSerres et al. 1993). It is the major constituent of native dermal extracellular matrix (collagen types I and III) and is therefore a natural choice. Burke et al (Burke 1981) used a bovine collagen based material to develop a dermal replacement product which has a poly-silicone "epidermal layer" to provide mechanical strength and act as a barrier to control the loss of moisture and prevent microbiological infection. Bell et al (Bell, Ehrlich et al. 1981) showed that a collagen lattice seeded with autologous skin fibroblasts formed dermal like tissue which was capable of supporting the differentiation of keratinocytes seeded onto these lattices.

Fibronectin, one of the dual function proteins, represents an important component of the ECM and is second only to collagen in quantity within the ECM. Fibronectin exists both in soluble and tissue forms and possesses many desirable properties of a tissue repair scaffold including ligands for adhesion of many cell types (Schwarzbauer and Sechler 1999). Fibronectin exists within the ECM of both submucosal structures and basement membrane structures. The fibronectin component of a commercially derived ECM scaffold derived from the porcine small intestinal submucosa has been shown to be

partially responsible for the adhesion of endothelial cells during *in-vivo* reconstructive modelling of this xenogenic bioscaffold. The dual characteristics (absorptive and cyto-adhesive) of this protein have made it attractive for use as a coating protein upon various synthetic scaffold materials to promote host biocompatibility (Ku, Chung et al. 2005).

Vitronectin is a glycoprotein present in plasma and tissues. Together with fibronectin, vitronectin is one of the major cell adhesion proteins of plasma. Although these proteins have similar functions and both have an Arg-Gly-Asp (RGD) cell recognition sequence binding $\alpha_v\beta_3$ and $\alpha_v\beta_5$, they are structurally and immunologically distinct. Found in the blood vessel basement membrane, vitronectin functions in several biological processes. In addition to promoting the adhesion of various cells in culture, vitronectin binds to glycosaminoglycans and is incorporated as an inhibitor to the membrane cytolytic attack complex of the complement system. It interacts with thrombin and antithrombin III during coagulation and may have a physiological role in the coagulation pathway as well as having a role in angiogenesis (Zhou 2003).

Laminins are a complex of adhesion proteins found in the ECM especially within basement membranes. Previous work in the laboratory has shown that both laminin and matrigel similarly induce differentiation of HPMEC into tubular structures, indicative of a vasculogenic phenotype distinct from the migration/invasion phenotype in collagen (Dye, Lawrence et al. 2004). Hence owing to practical limitations, matrigel (GF depleted) was used in this thesis as a crude form of laminin. Matrigel is a matrix of mouse basement membrane neoplasm consisting of a complex mixture of basement membrane proteins including type IV collagen, entactin/nitrogen and heparan sulphate but principally it contains laminin (>60%). Laminins are trimeric cross linked polypeptides that exists in numerous isoforms dependent upon the particular peptide chains (α1, β1 γ1) (Timpl 1996). These large molecules promote cellular adhesion and migration via integrins and other cell surface receptors and thus play a key role in tumour angiogenesis (Engbring and Kleinman 2003). The various isoforms have a cell and tissue specific expression and are differentially recognised by integrins (Patarroyo, Tryggvason et al. 2002). As the fundamental component of the basement membrane, laminin provides specific adhesion sites for cells which provides signals and regulates vessel homeostasis. It is commonly used as an ECM for *in-vitro* assays of endothelial cellular migration and angiogenesis (Benelli 1999). It is thus known for its ability to promote capillary formation and cellular invasion (Benelli 1999). Laminin 10 subtype has been isolated as a key ECM in wound

healing. Laminin 8 has been shown to promote dermal endothelial cell attachment, migration and tubule formation (Li, Zhang et al. 2003). Laminin has many active biological sites, principally RGD, YIGSR and SIKVAV. These, *in-vitro*, respectively promote cell binding, alterations in cell morphology and induction of migration and collagenase activity. The angiogenic effect of these sites contrasts greatly. The YIGSR inhibits both tumour growth and angiogenesis. SIKVAV alternatively initiates angiogenesis and tumour progression (Grant 1994). The prominent role of laminin in the formation and maintenance of vascular structures is particularly noteworthy when considering the ECM as a scaffold for tissue repair. Vascularisation of scaffolds for tissue repair is one of the rate limiting steps in the field of tissue engineering and laminins are receiving close attention as important components of endothelial cell specific scaffold materials (Doi, Thyboll et al. 2002; Oyane, Uchida et al. 2005).

The most abundant heteropolysaccharides in the body are the glycosaminoglycans (GAGs). These molecules are long unbranched polysaccharides containing a repeating disaccharide unit. The disaccharide units contain either two modified sugars N-acetylgalactosamine (GalNAc) or N-acetylglucosamine (GlcNAc) and an uronic acid such as glucuronate or iduronate. GAGs are highly negatively charged molecules, with extended conformation that imparts high viscosity to the solution. GAGs are located primarily on the cell surface and in the extracellular matrix (ECM). GAGs form high viscosity solutions and strongly bind to water which forms a hydration sheath with lubricating and bulking properties. At the same time, their bulk provides structural integrity to the ECM and provides passageways between cells, allowing for cell migration. The specific GAGs of physiological significance are hyaluronic acid, dermatan sulphate, chondroitin sulphate, and heparan sulfate. Although each of these GAGs has a predominant replicating disaccharide unit, heterogeneity does exist in the sugars present in the make-up of any given class of GAG. The heparin binding properties of numerous cell surface receptors and of many growth factors (FGF family, VEGF) make the heparin rich GAGs desirable components of scaffolds for tissue repair.

3.1.2 ECM and growth factors

The ECM has been found to sequester significant amounts of growth factors and cytokines and their release under specific circumstances can have potent effects on cell behaviour. The list of such growth factors is extensive and includes VEGF, bFGF, EGF,

TGFβ, KGF, HGF and PDGF (chapter 1.4). Many of these factors are known to exist in multiple isoforms, each with its specific biologic activity. Purified forms of growth factors and biologic peptides have been investigated in recent years as therapeutic means of encouraging blood vessel formation, stimulating granulation tissue and wound epithelialisation. However this therapeutic approach has struggled with determination of optimal dose, sustained and localised release at the desired site and the inability to turn the factor "on" and "off" as needed during the course of tissue repair. A theoretical advantage of utilising the ECM in its native state as a scaffold for tissue repair is the presence of all the attendant growth factors (and their inhibitors), in the relative amounts that exist in nature and perhaps importantly in their native three dimensional ultra-structure. Some bioengineered scaffolds may mimic this, through the incorporation of fibroblasts within their scaffold to produce a cocktail of growth factors (Dermagraft and Apligraft).

3.1.3 ECMs for angiogenesis in bioengineered dermal replacements

Early studies into the generation of bioengineered synthetic dermal equivalents focussed on principals such as;

> Chemical integrity
> Mechanical strength and porosity
> Pliability
> Proteolytic stability
> Biocompatibility (Yannas and Burke 1980)

While these properties are essential in the design of a matrix, recent studies have investigated the potential to increase take of such bioengineered matrices. As explained in the introduction (chapter 1.3.3.6) a crucial component of take is the viable incorporation and invasion of endothelial cells into the matrix, stimulating neovascularisation. This key component of angiogenesis is essential in the bio-integration of a successful dermal graft (Converse 1975).

The extracellular matrix is thought to play a crucial role in endothelial cell reorganisation and behaviour during angiogenesis (Folkman and Klagsbrun 1987). Changes in the composition of the ECM have also been shown to be responsible for the survival of endothelial cells (Iruela-Arispe, Diglio et al. 1991). However the role of the ECM signals

in regulating angiogenesis is complex and poorly understood. It is well established that different matrices induce various alternative patterns of endothelial response with cellular adhesion via integrins and other receptors being essential for these responses with matrix specific remodelling. Recent studies have demonstrated that gel strength dictates the degree of endothelial cell sprouting, invasion (Korff and Augustin 1999) and tubulogenesis (Deroanne, Lapiere et al. 2001), these responses being integrin receptor mediated (Davis and Camarillo 1996). Other receptors also play a distinct role in the migration of endothelial cells such as the 60Kd laminin binding protein and the recently identified scavenger/laminin fragment binding protein.

Such possible cellular mechanisms leads to the basic hypothesis that the ECM may function not simply as a "bio-compatible" adhesive support for cells, but may supply signals which stimulate angiogenesis and other cell-specific responses (Dye, Lawrence et al. 2004). Such effects may be critical for the design of a bio-engineered matrix.

Although various ECM constituents have been found to exert "angiogenic-type" effects as outlined above, it is not clear from the literature whether any of these effects would translate into sufficient stimulation, of endothelial cell ingress, to be surgically useful for a synthetic scaffold. Therefore this chapter aims to investigate and compare the potential of ten different extracellular matrices to induce a change in phenotype from resting to invasive migrating endothelial cells and quantitatively compare this response using an *in-vitro* assay (Nehls and Drenckhahn 1995) that allows the analysis of matrix proteins and soluble angiogenic stimulants. The assay uses human endothelial cells cultured on an inert carrier, allowing invasion of a given matrix from a fixed point (chapter 2.2.4). This assay was conceived as an angiogenic assay (Nehls and Drenckhahn 1995), variations of which have been used by other groups studying angiogenesis. Close correlation has been demonstrated between *in-vitro* 'angiogenic' morphologenesis and *in-vivo* angiogenesis in the chick chorioallantoic membrane system (Bootle-Wilbraham 2001).

3.2 Materials & Methods

3.2.1 Cell Culture

Human placental microvascular endothelial cells were cultured from human placentae as previously described (chapters 2.2.1 – 2.2.3).

3.2.2 Cytodex bead assay

Cytodex beads™ (Pharmacia Biotech) were prepared and cultured with endothelial cells as described (chapter 2.2.4). At least three cell lines from different placentae were used per experiment. Endothelial cell migration assays were established suspending confluent beads in ECM gels. The gels were incubated with equal quantities of minimal medium (MM, defined in chapter 2.2.3) for 96hrs with all media exchanged at 48hrs. Matrices were fixed at 96 hours and cellular invasion of the matrices measured using sigma scan (chapter 2).

Mean migration distances were calculated, and it was found that relative variations in absolute invasion occurred between cell isolates and experiments. All migration values were therefore expressed as a ratio to migration in 0.75 mg/ml collagen (MM migration given a value of 1). This concentration was found to give an optimal migration for collagen alone. Data was analysed by one way ANOVA with Tukey post-hoc test (multiple comparison).

The following variable experimental conditions were explored;
  1) Collagen gels

Rat-tail collagen was prepared using MM at concentrations of 0.5 0.75, 1, 1.5 and 2.0 mg/ml (n=4) to obtain the optimal concentration for migration.

  2) Two-matrix composite collagen gels

Based on the optimal concentration of collagen (0.75mg/ml), matrices were manufactured using equal volumes of collagen at double concentration (1.5mg/ml) plus each of the matrices in table 3.1 at double their respective final concentrations.

Table 3.1 Concentrations of Extracellular Matrices used and investigated for their influence on endothelial cell migration

Extracellular Matrix	Final Concentrations
Fibrinogen Fraction 1	0.5, 1.3, 5 mg/ml
Vitronectin	0.01, 0.1, 1 µg/ml
Fibronectin	2, 6, 20 µg/ml
Laminin (matrigel, growth factor reduced))	0.1875, 0.375, 0.75 mg/ml
Elastin (bovine neck ligament)	0.1, 0.3, 1 mg/ml
Hyaluronic acid (human umbilical cord)	0.1, 0.3, 1 mg/ml
Dermatan Sulphate	0.1, 0.3, 1 mg/ml
Chondroitin Sulphate A (bovine trachea)	0.1, 0.3, 1 mg/ml
Heparan Sulphate sodium salt (bovine kidney)	0.01, 0.03, 0.1 mg/ml

3) Multiple collagen/fibrin concentration gels

Collagen was prepared using MM at concentrations of 1.0, 1.5 and 3mg/ml. Fibrinogen was prepared at 10, 2.6 and 1 mg/ml. Combinations of each concentration of each of the two matrices were used to give the nine permutations as shown in figure 3.8. Collagen 0.75mg/ml served as a control. Fibrinogen was also prepared at 5, 1.3 and 0.5 mg/ml for use without collagen.

4) Triple matrix composite collagen/fibrin gels

Triple matrices were made using the optimum concentrations of collagen and fibrin plus one of the following extracellular matrices over the indicated concentration ranges.

Chondroitin	0.1, 0.3, 1 mg/ml
Elastin	0.1, 0.3, 1 mg/ml
Hyaluronan	0.1, 0.3, 1 mg/ml

Matrigel (laminin)	0.1875, 0.375, 0.75 mg/ml
Vitronectin	0.01, 0.1 1 µg/ml

Table 3.2 The matrices added to a collagen/fibrin suspension

All matrix components (collagen, fibrin and matrices shown in table 3.2) were prepared at triple strength, thus diluted to a third strength on mixing together. All dilutions from concentrated preparations were made with MM.

5) Extraction of the soluble component of collagen and angiogenic assessment

Collagen supernatant was prepared as outlined (chapter 2.2.8). Collagen (0.75mg/ml), once gelled, was incubated with MM or the collagen supernatant for 96 hours. All media was exchanged at 46 hours. Gels were fixed at 96 hours and cellular invasion assessed as per experiments 1-4.

3.3 Results

3.3.1 Collagen induces angiogenic sprouting of microvascular endothelial cells

Culture of confluent endothelial cells on cytoballs with collagen over a range of collagen gel concentrations consistently caused endothelial cell sprouting, substratum detachment and three-dimensional invasion in line with previous in-house studies (figure 2.6 lower) (Dye, Lawrence et al. 2004). This is indicative of a switch from quiescent to invasive behaviour. This behaviour was consistent in all cell isolates studied. Endothelial cell invasion displayed a biphasic variation with collagen concentration, with an optimum migration at 0.75 mg/ml ($p < 0.001$, ANOVA, 0.75mg/ml and 0.5, 1.0, 1.5 and 2mg/ml, n=4 cell lines). Mean invasion distance of pooled data from all experiments over 96 hrs for 0.75 mg/ml was 71.3 microns. All further collagen gels were used at the optimum concentration of 0.75 mg/ml unless otherwise stated.
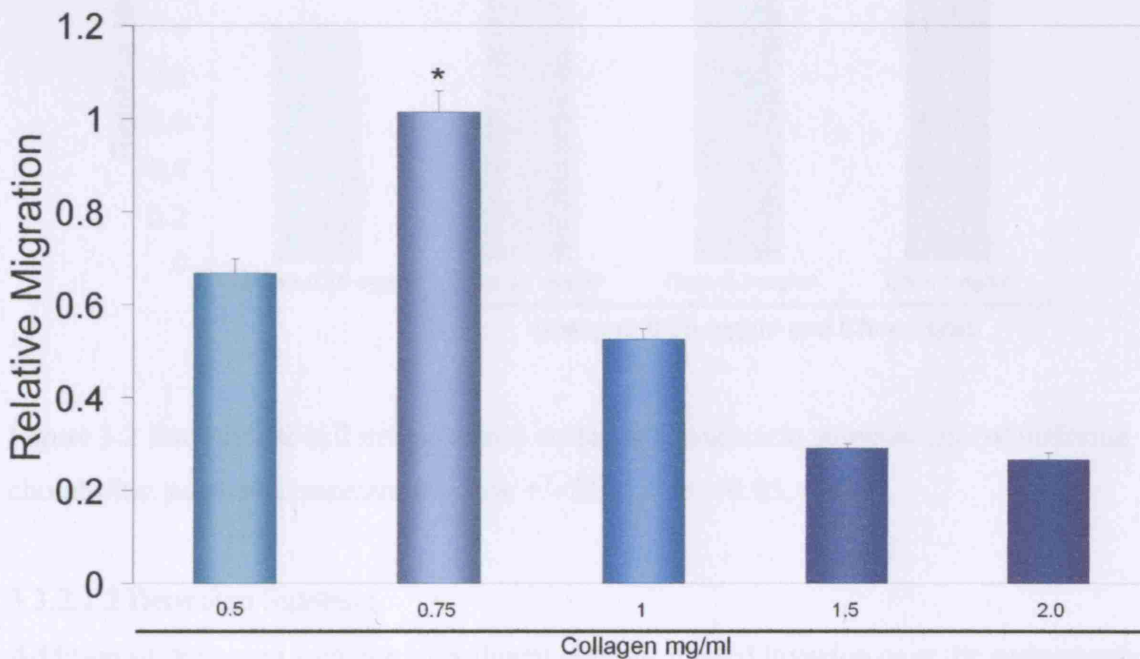
Figure 3.1 endothelial cell migration within collagen gels of differing concentration (mn +/- SEM, * p<0.001, n=4)

3.3.2 Angiogenic invasion into two matrix composite collagen gels

3.3.2.1 GlycosaminoGlycans

3.3.2.1.1 Chondroitin Sulphate

Addition of chondroitin sulphate to collagen at all concentrations studied stimulated an increase in migration over collagen alone, ($p < 0.05$, Tukey, n=4). Although there was no difference statistically between the concentrations ($p > 0.05$, Tukey), the increase in migration was maximal at 1 mg/ml, achieving 1.79 times that of collagen alone (table 3.3). Mean invasion distance of pooled data from all experiments over 96 hrs for 1 mg/ml was 55.67 +/- 50.23 microns.
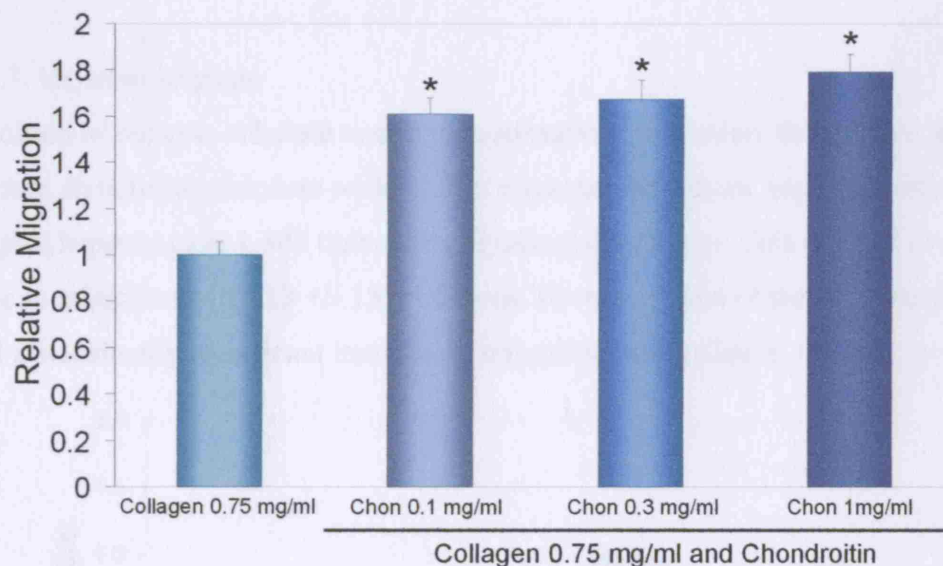
Figure 3.2 Endothelial cell invasion into collagen/chondroitin sulphate gels of differing chondroitin sulphate concentration (mn +/- SEM, * $p < 0.05$, n=4)

3.3.2.1.2 Dermatan Sulphate

Addition of dermatan sulphate to collagen gels stimulated invasion over the concentration ranges studied although this was not as much as with chondroitin. The strongest invasive response, seen with 1mg/ml, giving an increase in migration of 28.79 +/- 5.41 microns (1.44 times that of collagen) did not reach statistical significance (p=0.082, n=3).
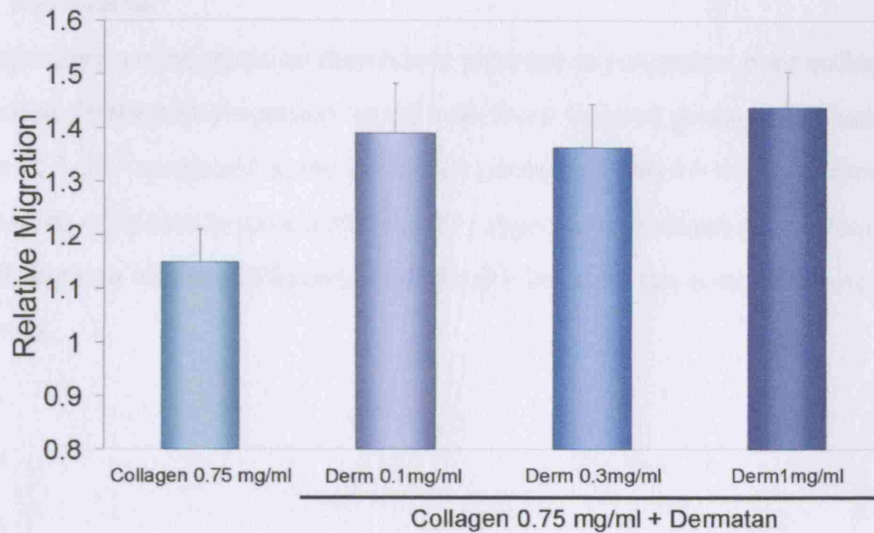
Figure 3.3 Endothelial cell migration in collagen gels with dermatan suphate (mn +/- SEM, n=3).

3.3.2.1.3. Heparan sulphate

The addition of heparan sulphate to collagen stimulated migration, though to a lesser extent than chondroitin sulphate or dermatan sulphate. Maximum migration from 0.03mg/ml heparan gave 1.303 times the migration of collagen. This equated to an increase in migration of 19.13 +/- 13.2 microns. However none of the concentrations yielded a statistically significant increase in migration over collagen (p = 0.5, n=3).
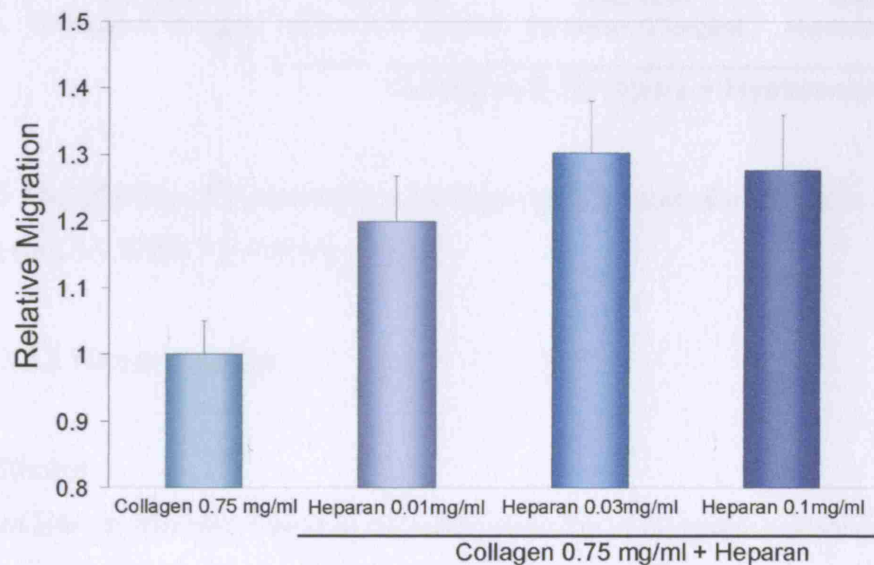
Figure 3.4 Endothelial cell invasion into collagen gels showing the minimal increase in invasion after adding heparan to the collagen matrices (mn +/- SEM, n=3)

3.3.2.1.4 Hyaluronan

Hyaluronan gave a concentration dependent increase in migration over collagen, over the range studied. Maximum migration found was from 1mg/ml giving an increase in migration of 1.337 compared to the collagen controls (45.46 +/- 41.13 microns). All three concentrations of hyaluron gave a statistically significant increase in migration. Nevertheless there was no difference statistically between the concentrations (p< 0.05, Tukey, n=3).
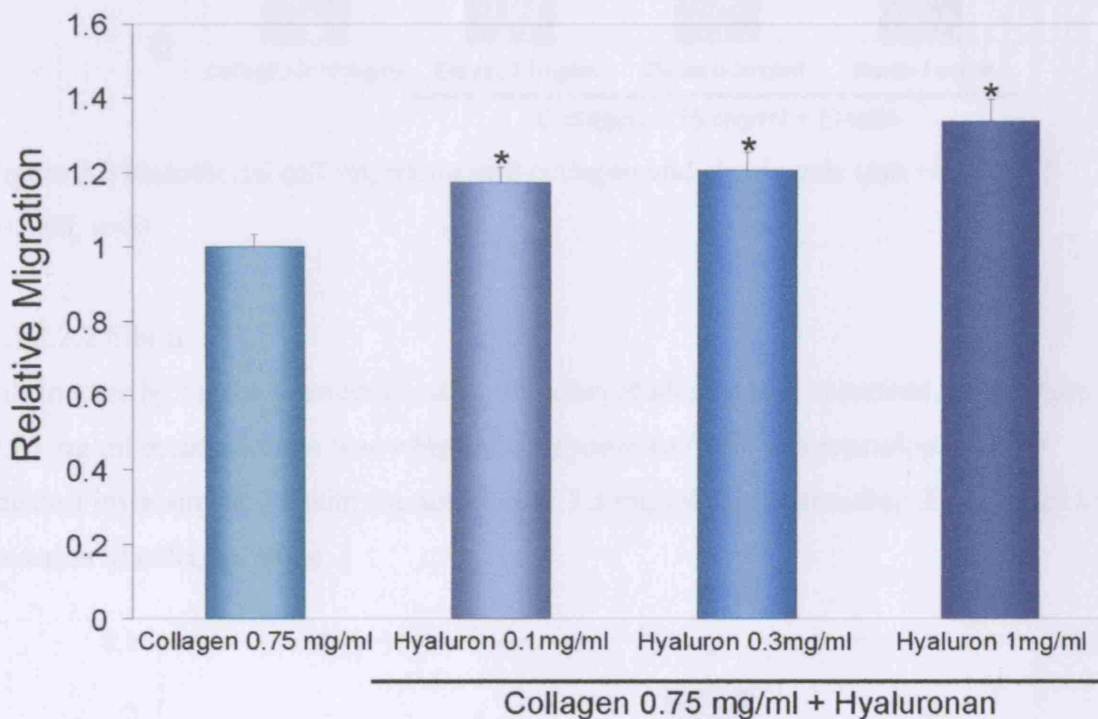
Figure 3.5 The addition of Hyaluronan to collagen gels increased endothelial cell migration (mn +/- SEM, * p < 0.05, n=3).

3.3.2.2 Plasma Proteins

3.3.2.2.1 Elastin

Addition of Elastin was also found to stimulate invasion at all concentrations studied compared to collagen (p< 0.05, Tukey, n=3). Greatest migration was at 1 mg/ml, giving 1.572 times the migration of collagen. There was no statistical difference between the three concentrations.
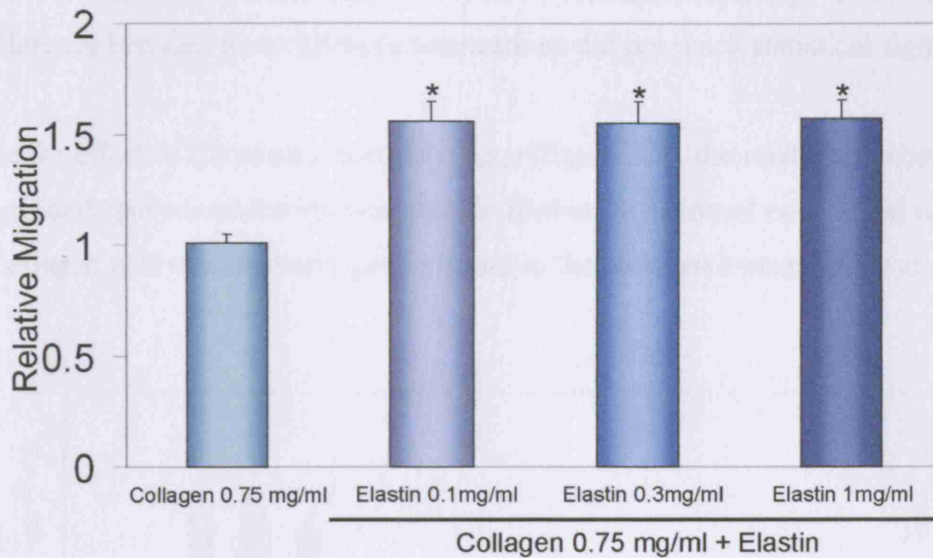
Figure 3.6 Endothelial cell migration into collagen and elastin gels (mn +/- SEM, * p<0.05, n=3)

3.3.2.2.2 Fibrin

Fibrin gave by far the greatest invasive stimulus of all matrices examined. In collagen 0.75 mg/ml matrices there was a biphasic response to fibrin concentration with the greatest invasion shown with the addition of 1.3 mg/ml fibrin providing 3.181 times the invasion of collagen alone.
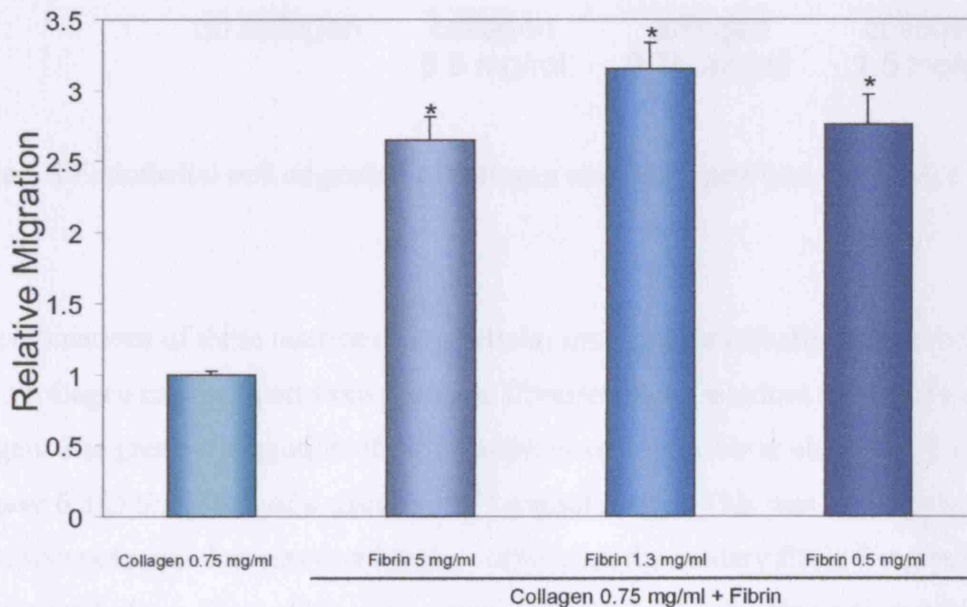
Figure 3.7 Endothelial cell migration within collagen gels (0.75 mg/ml) with the addition of fibrin (0.5 – 5 mg/ml, mn +/- SEM, * p < 0.05, n=7)

This equates to an increase in migration of 165 +/- 120.22 microns (p< 0.05, Tukey, n=7). The difference between these fibrin concentrations did not reach statistical significance.

Because the effect of fibrin was relatively large (figure 3.7), the relationship between fibrin and collagen concentration was studied further. Invasion of endothelial cells into fibrin/collagen gels was inversely proportional to the collagen concentration (n=3, figure 3.8).
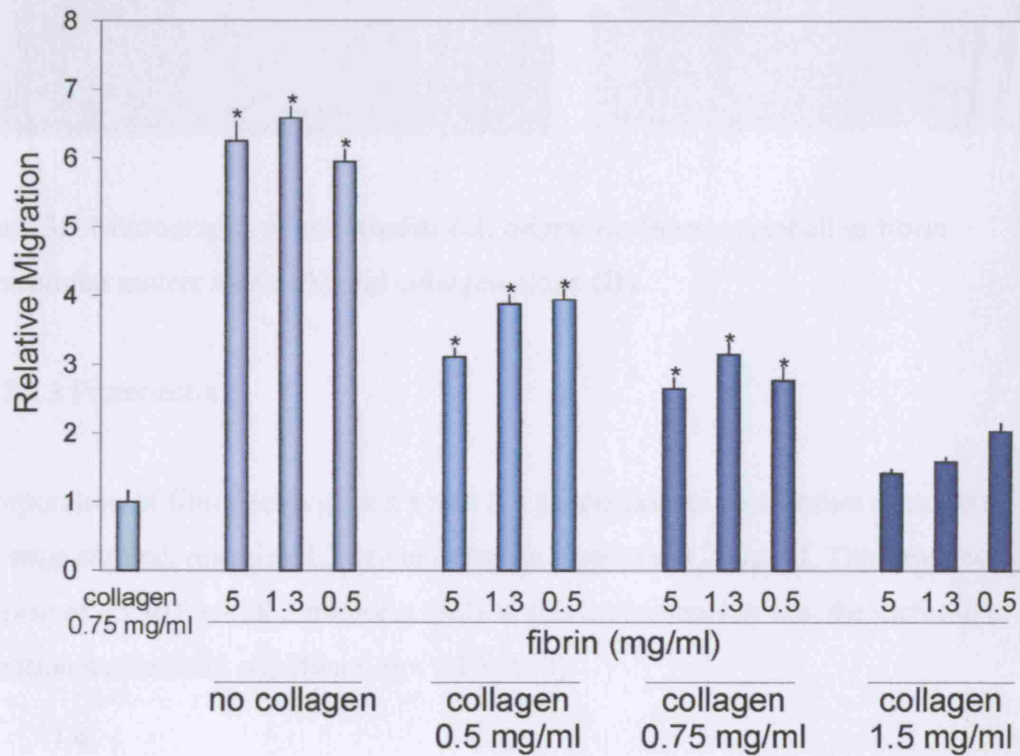
Figure 3.8 Endothelial cell migration in collagen and fibrin gels (mn +/- SEM, p < 0.05, n=3)

All combinations of these matrices gave cellular invasion statistically greater than a 0.75 mg/ml collagen matrix, apart from the three fibrin/collagen matrices using 1.5 mg/ml collagen. The greatest migration of these matrices was from fibrin alone at 1.3 mg/ml, this gave 6.583 times that of a collagen 0.75 mg/ml matrix. This was statistically greater in invasive potential than any combination other than the solitary fibrin 5 mg/ml. All matrices made from fibrin alone were statistically more invasive than any matrix including collagen (p< 0.05 Tukey). Owing to these results all fibrin matrices for future studies were made at the optimal fibrin concentration (1.3mg/ml). Migration in fibrin

demonstrated characteristics of vacuolation and capillary tip formation.
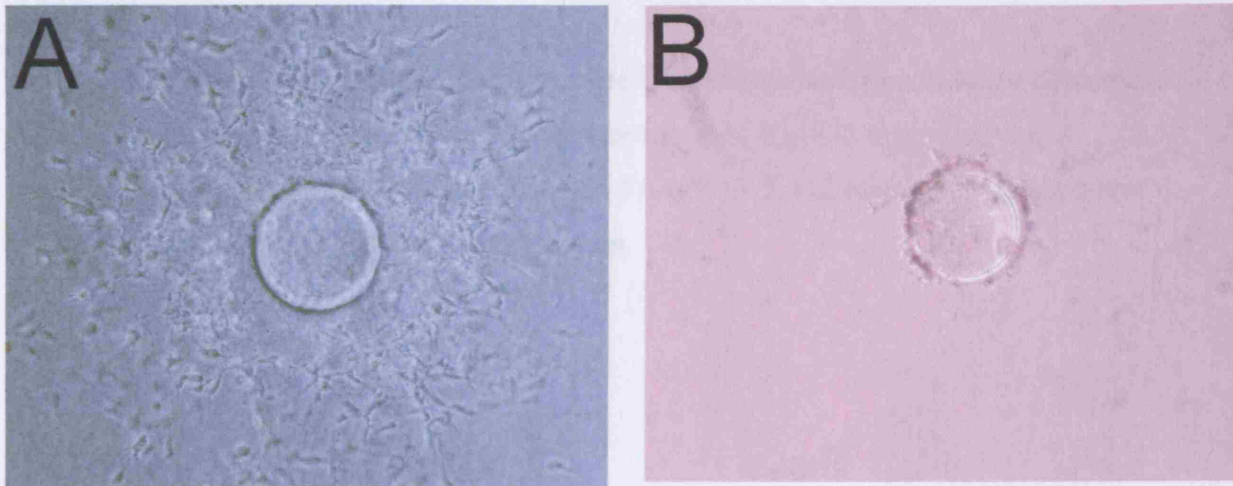
Figure 3.9 Micrographs of endothelial cell migration from a cytoball in fibrin extracellular matrix alone (A) and collagen alone (B).

3.3.2.2.3 Fibronectin

Incorporation of fibronectin gave a small but proportionate stimulation of migration over the range studied, reaching 1.32 over collagen matrices at 20μg.ml. This equated to an increase of 45.301 +/- 28.2 microns. Only at this concentration was the increase in migration statistically significant ($p < 0.05$, n=3).
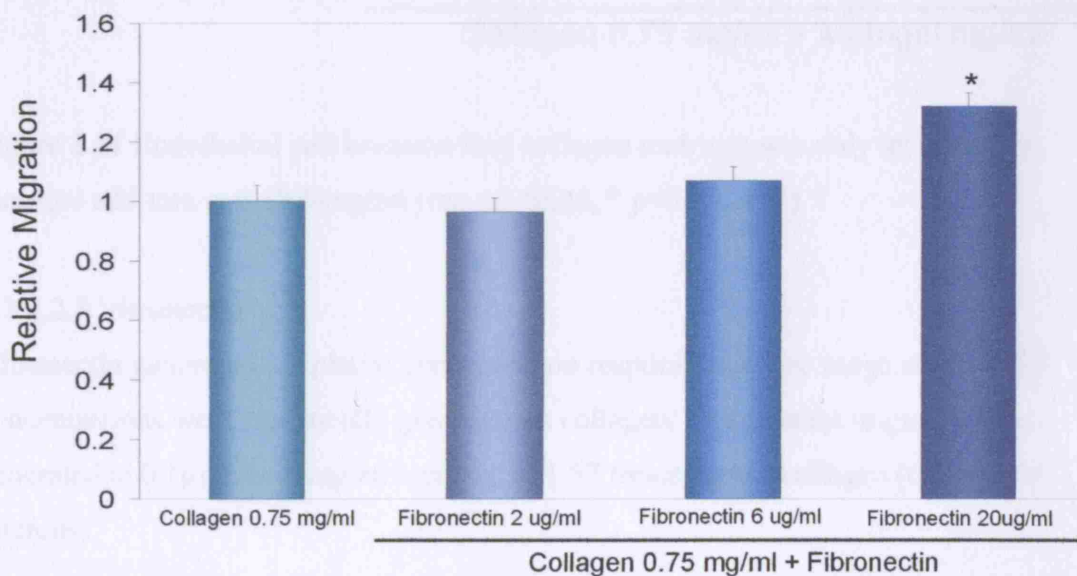
Figure 3.10 Endothelial cell migration in collagen gels with fibronectin added at varied concentrations (mn +/- SEM, * $p<0.05$, n=3).

3.3.2.2.4 Matrigel

Matrigel (composed principally of laminin, type IV collagen and growth factor depleted) increased migration only at the lowest concentration used (0.1875 mg/ml) giving a migration coefficient of 1.32, which equates to 51.449 +/- 2.812 microns. Migration was inversely proportional to matrigel concentration.
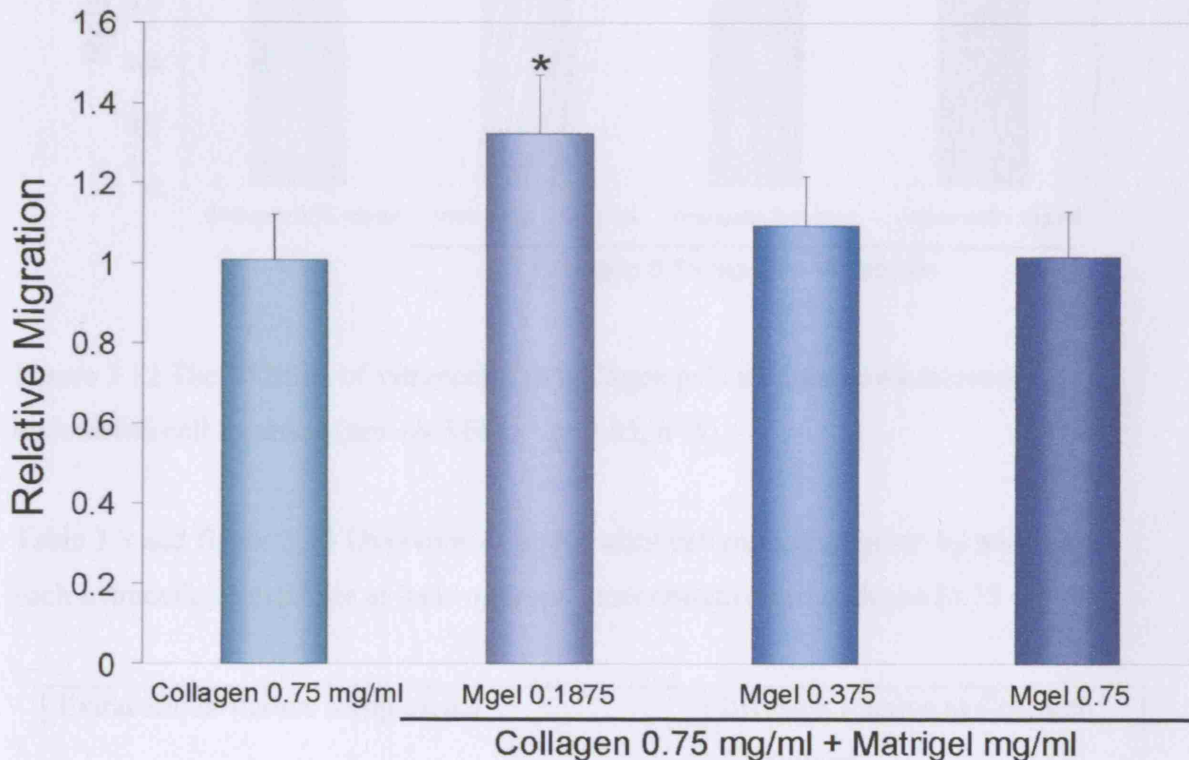
Figure 3.11 Endothelial cell invasion into collagen matrices was only increased by matrigel addition at 0.1875 ng/ml (mn +/- SEM, * $p < 0.05$, n=3).

3.3.2.2.5 Vitronectin

Vitronectin generated a biphasic concentration response over the range studied. All concentrations were statistically greater than collagen. The greatest migration was generated at 0.1µg/ml giving an increase of 1.57 times that of collagen (60.1 +/- 44.1 microns).
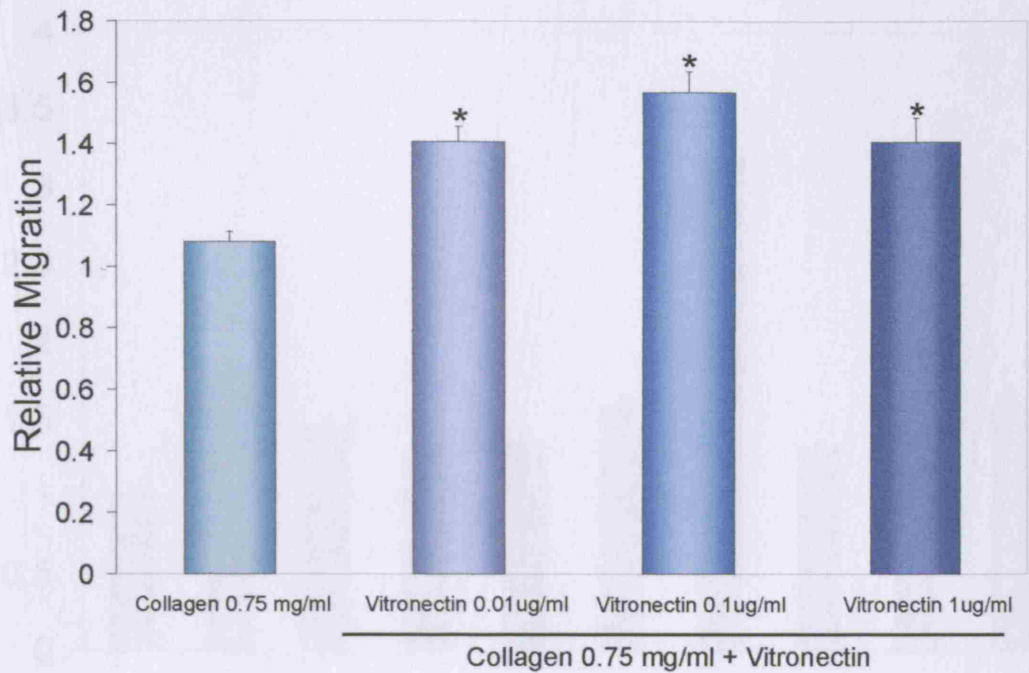
Figure 3.12 The addition of vitronectin to collagen gels stimulated an increase in endothelial cell invasion (mn +/- SEM, * p<0.05, n=5).

Table 3.3 and figure 3.13 Overview of endothelial cell migration given by addition of each extracellular matrices at their optimum concentrations to collagen (0.75 mg/ml)

Extracellular matrix components	Invasion relative to Collagen 0.75mg/ml
Chondriotin sulphate and collagen 0.75 mg/ml	1.790
Dermatan sulphate and Collagen 0.75 mg/ml	1.437
Heparan sulphate and collagen 0.75 mg/ml	1.303
Hyaluron and collagen 0.75mg/ml	1.337
Elastin and Collagen 0.75mg/ml	1.572
Fibrin and collagen 0.75 mg/ml	3.181
Fibronectin and collagen 0.75 mg/ml	1.320
Matrigel (GF reduced) and collagen 0.75 mg/ml	1.320
Vitronectin and collagen 0.75 mg/ml	1.567
Fibrin 1.3 mg/ml	6.903

Collagen 0.75 mg/ml	Dermatan 1%	Hyaluronan 1mg/ml	Fibrin 1.3 mg/ml	Mgel 0.1875 mg/ml
Chondroitin 1mg/ml	Heparan 0.03 mg/ml	Elastin 1 mg/ml	Fibronectin 20ug/ml	Vitronectin 0.1ug/ml
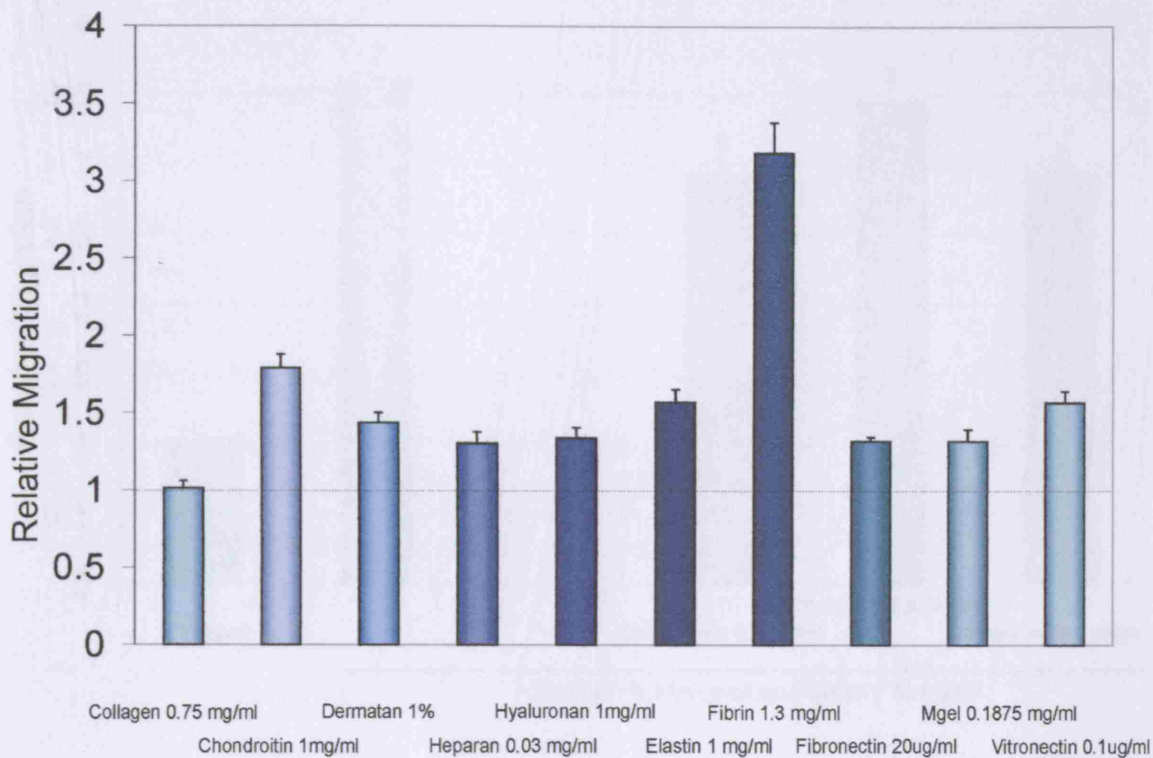
3.3 Three matrix composite collagen fibrin gels

Using the optimum fibrin and collagen concentrations (1.3 and 0.75 mg/ml respectively) five other ECMs were admixed to investigate whether their addition would further stimulate migration. In all cases migration decreased when a third matrix was added.

3.3.1 Chondroitin

Chondroitin attenuated migration with a biphasic relationship to concentration, distinct from the collagen/chondroitin matrices which increased migration in response to increasing chondroitin concentration. The attenuation of infiltration was about 1% less than that of fibrin/collagen matrices alone (statistically insignificant ($p > 0.05$, Tukey, n=3). Maximum migration with 0.3mg/ml was 3.453 times the migration of the control collagen gel, yet this was 0.15 less than the collagen/fibrin admixture. All triple chondroitin matrices gave a statistically significant increase in migration over collagen gels. There was no difference between the three chondroitin concentrations ($p > 0.05$).

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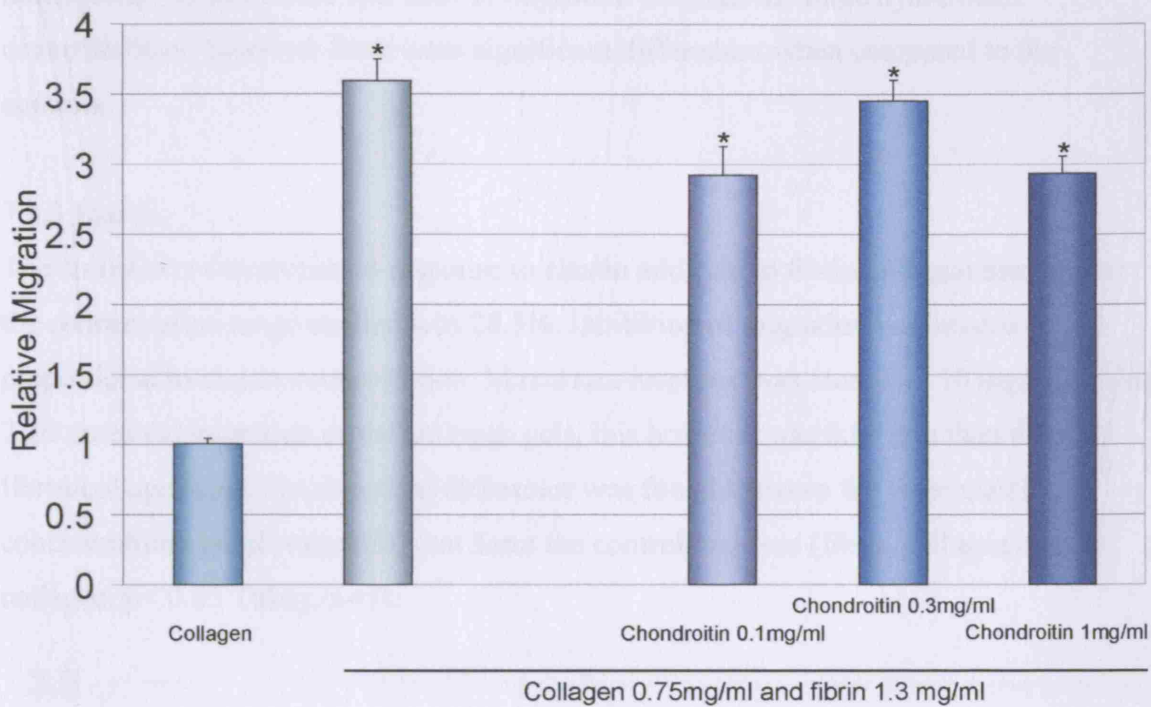
Figure 3.14 Addition of chondroitin to fibrin collagen gels inhibited endothelial cell invasion (mn +/- SEM, p<0.05 vs collagen, n=3)

3.3.2 Hyaluronan

Addition of hyaluronan attenuated migration at all concentrations tested by about 30% of the fibrin/collagen control. This contrasts with its linear enhanced migration response when added to collagen alone. 0.3 mg/ml yielded the greatest migration being 2.7 times that of collagen, this was 0.751 less than the fibrin control.
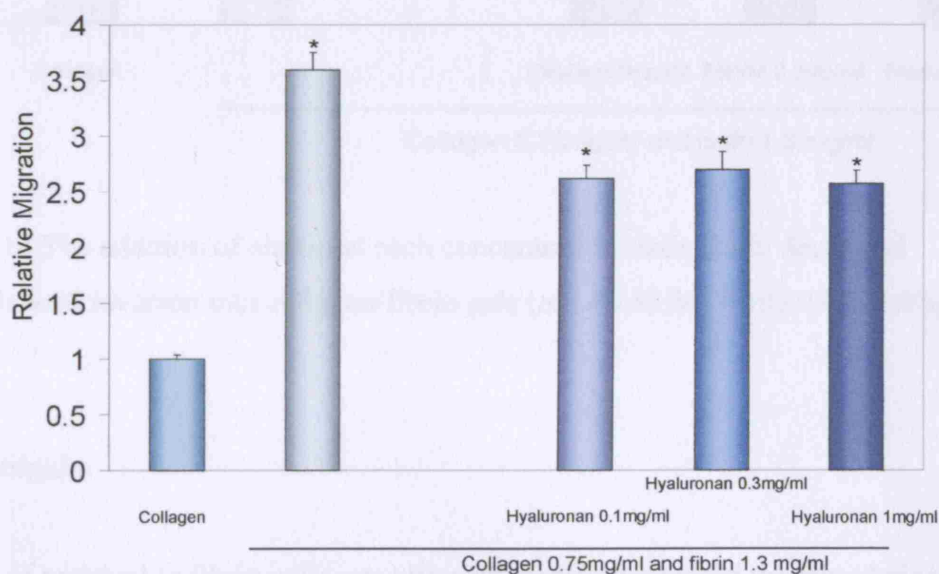
Figure 3.15 The attenuation of endothelial cell migration in fibrin/collagen gels after the addition of hyalorunan (mn +/- SEM, * p<0.05 vs collagen, n=3)

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Statistically, no difference was seen in migration between the three hyaluronan concentrations. However there were significant differences when compared to the controls.

3.3.3 Elastin

The reduction of migration in response to elastin addition to fibrin/collagen matrix over the concentration range studied was 28.5%. Inhibition of migration was inversely proportional to elastin concentration. Maximum response was seen with 10 mg/ml, giving 2.20 times the migration of the collagen gels, this however was 0.88 less than the fibrin/collagen gels. No statistical difference was found between the three elastin concentrations yet all were different form the control matrices (fibrin/collagen and collagen, p< 0.05 Tukey, n=3).

Collagen 0.75mg/ml and fibrin 1.3 mg/ml
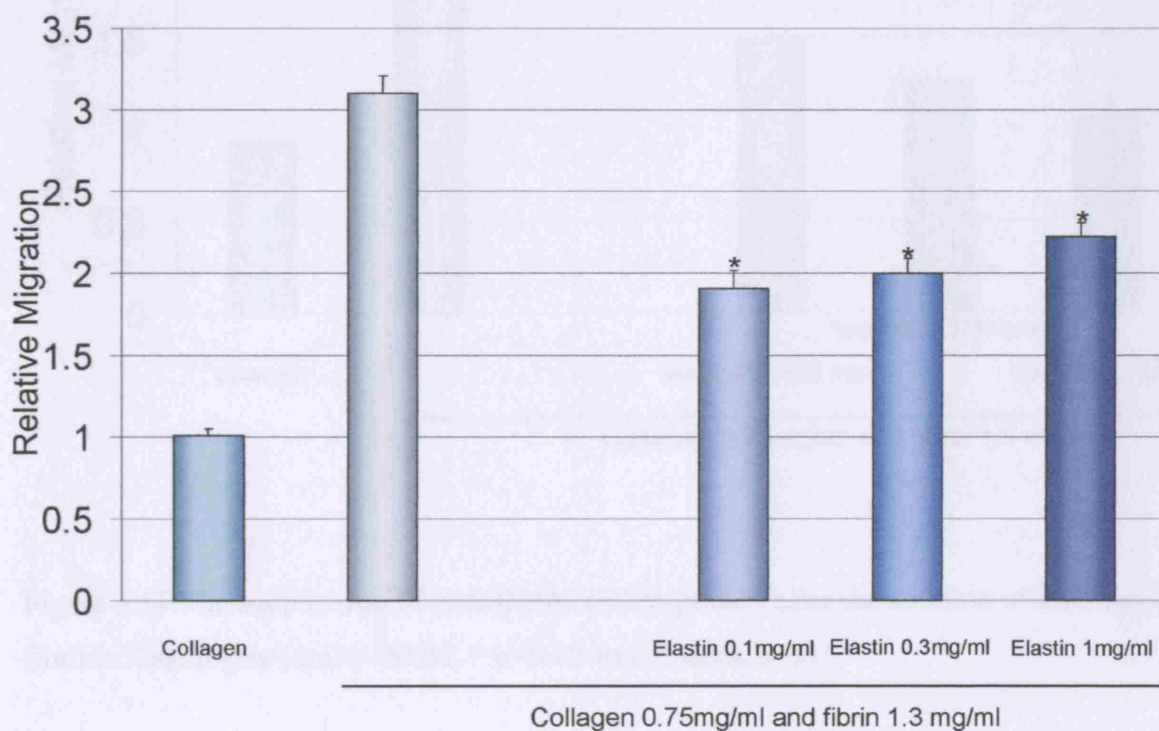
Figure 3.16 The addition of elastin at each concentration statistically decreased endothelial cell invasion into collagen/fibrin gels (mn +/- SEM, * p<0.05 vs collagen, n=3).

3.3.4 Matrigel

Addition of matrigel to fibrin collagen gels profoundly suppressed the stimulating effect of fibrin over the range studied. The response was similar to that with collagen/matrigel

matrices. Maximum migration was seen with 0.1875 mg/ml giving 1.477 times that of collagen yet this was 1.2504 less than the fibrin collagen matries. No difference was seen between the collagen control and the 0.75 mg/ml matrigel fibrin collagen matrix (p>0.05). The addition of matrigel to the fibrin collagen matrices significantly decreased migration (Tukey, p<0.05, n=3)
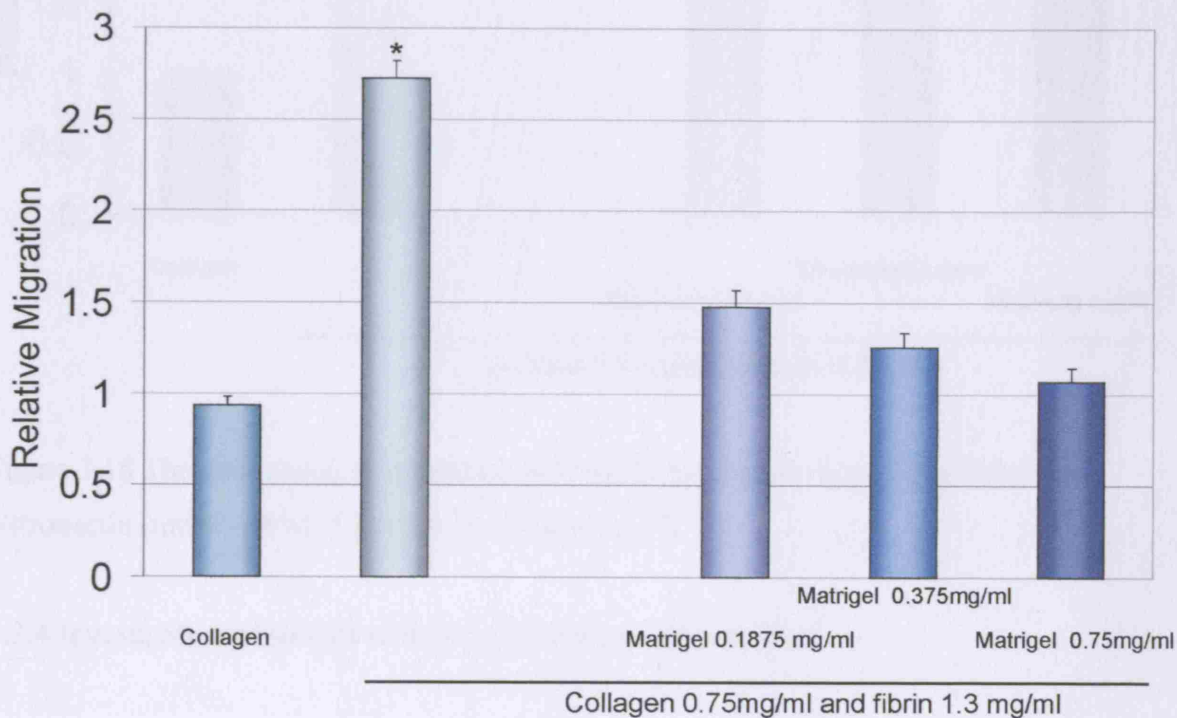
Figure 3.17 The suppression of endothelial cell migration after the addition of matrigel to fibrin/collagen gels (mn +/- SEM, * p<0.05 vs collagen, n=3).

3.3.5 Vitronectin

Vitronectin in the triple matrices also markedly suppressed the stimulating effect of fibrin by 36%. Maximum migration was with 0.1μg/ml, this was 1.24 times the fibrin/collagen control. No difference was seen between the vitronectin concentrations, but all were less than the fibrin/collagen controls (Tukey, p<0.05, n=3).
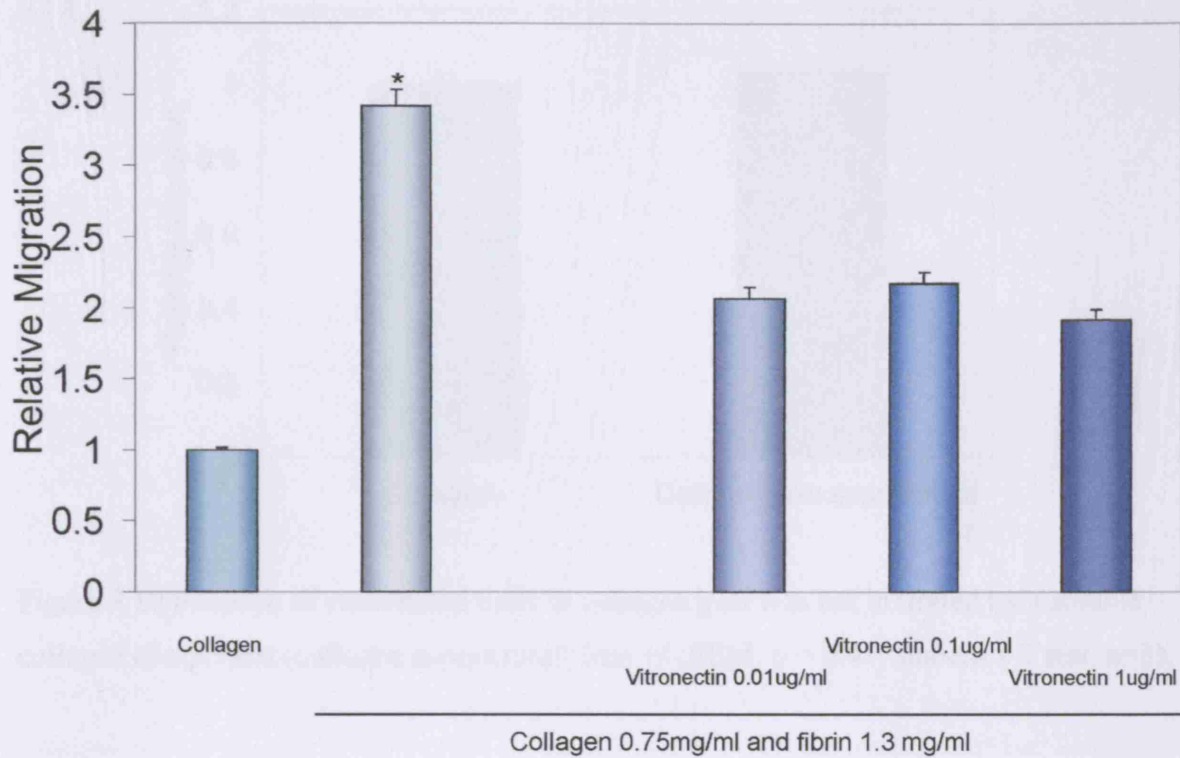
Figure 3.18 The attenuation of migration in fibrin/collagen gels from the addition of Vitronectin (mn +/- SEM, * p<0.05 vs collagen, n=3).

3.3.4 Investigation of soluble collagen gel products on invasion

Because the cell morphology in collagen was suggestive of migration, yet this was low, the possibility that invasion in collagen was being suppressed by a soluble fraction from the collagen, rather than the collagen matrix itself was investigated. Collagen supernatant was added to cytoballs in collagen, replacing the MM. Invasion was compared to collagen with stimulation from MM. There was no difference in invasion between MM and collagen supernatant, suggesting that the collagen matrix, rather than soluble salts or peptides, was responsible for the limited invasion in collagen (p = 0.41, student's T test, n=3).
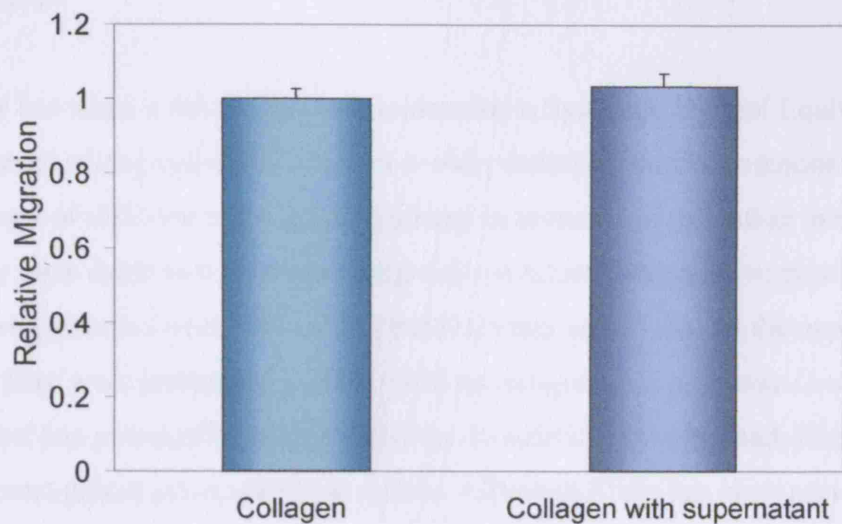
Figure 3.19 Invasion of endothelial cells in collagen gels was not inhibited by a soluble collagen component (collagen supernatant) (mn +/- SEM, p = 0.41, student's T test, n=3).

3.4 Discussion

This study has taken a novel approach to develop a Synthetic Dermal Equivalent using an *in-vitro* model of angiogenesis to screen a wide variety of matrix components. Comparisons of different matrices on angiogenic invasion or migration have not previously been made in conditions designed to evaluate comparative matrix effects distinct from growth factor effects. It should be emphasised that all the experiments described here were performed in MM, without exogenous angiogenic Growth Factors. This chapter has principally found that of the 10 matrices investigated, fibrin was found to be the most potent pro-angiogenic matrix. Although fibrin has been previously identified as a matrix in which angiogenesis physiologically occurs, it's specific effects on endothelial invasion have not previously been explored.

Within *in-vitro* and *in-vivo* settings collagens have been found to promote cell-specific attachment, migration, proliferation and differentiation (Ojeh, Frame et al. 2001). Studies have shown this to be true for fibroblast invasion (Vaissiere 2000). Also previous work using an *in-vitro* endothelial cell model indicated that collagen promotes a switch to a migratory phenotype (Dye, Lawrence et al. 2004). The data in this chapter is surprising in showing that endothelial cell migration, though somewhat supported by collagen, is markedly limited. This suggests that the disrupted morphology of cells in collagen compared to a regular endothelial monolayer is distinct from active migratory behaviour. The biphasic concentration response of migration would be consistent with opposing effects of physical gel rigidity and density with adhesion site concentration for cell attachment or migration being a function of both attachment and sprouting. The analysis of collagen supernatant (chapter 3.3.4) showed that such limited migration is an intrinsic property of the collagen matrix rather than of a soluble component, such as an RGD containing fragment, which may have caused de-adhesion or induced apoptosis (Buckley, Hill et al. 1988).

In acute wound healing fibrin functions as the initial structural scaffold for adhesion, proliferation and migration of cells in the healing wound. Fibrin has been successfully used as a treatment for critical ischaemia in animal and human trials (Chekanov, Rayel et al. 2002; Kipshidze, Kipiani et al. 2003). As a component of a Synthetic Dermal Equivalent it has been shown to improve the regeneration of a mature epidermal structure

(Hojo, Inokuchi et al. 2003) and stimulate vascular invasion (Kroon, van Schie et al. 2002; Llames 2004).

As a structural protein it serves to promote endothelial cell invasion and thus angiogenesis. Fibrin has been established as having a role in angiogenesis as a provisional matrix if not permissive scaffold, through RGD sites. Previous work has identified Fibrin Degradation Products (FDPS) as a possible mechanism for stimulating invasion from tumour angiogenesis and fibrin degradation and plasminolysis. Numerous mechanisms of action have been postulated for fibrin's action, most centre on the binding of fibrin to cells. Platelets bind to fibrin by the sequence 400-411 of the extreme C terminus of the γ chain) (Cheresh, Berliner et al. 1989). Cells such as endothelial cells, fibroblasts and smooth muscle cells bind to fibrinogen/fibrin through cell integrins (Yee, Rooney et al. 1998), the proposed binding domains being the RGD epitopes at locations α 95-97 and α 572-574. Recent research has identified novel binding Haptotactic motifs that exist in the C termini of the ß chain as well as near the C terminus of the γ chain and the extended αE chain (Gorodetsky, Vexler A et al. 2003). While specific binding sites have provided a mechanism for endothelial cell interaction and angiogenesis, research on fibrin subunits and degradation products has shown the pro-angiogenic poperties of fibrin subunit E (Bootle-Wilbraham 2001).

In this chapter fibrin has been identified as an alternative matrix protein with a more potent pro-invasive angiogenic effect than collagen, independent of soluble angiogenic factor stimuli. The effect of fibrin in collagen matrices was greater than any other matrix or soluble angiogenic factor tested. The addition of collagen to fibrin matrices inhibited cellular invasion. Fibrin previously has been shown to serve as a pro-angiogenic protein; it induces endothelial cell sprouting in *in-vitro* angiogenic models (Sieminski 2004) and stimulates tubulogenesis, with concomitant MMP and MT-MMP action (Collen, Hanemaaijer et al. 2003). It plays a pivotal role in the formation of capillary-like tubular structures due to its mechanical properties and following fibrinolysis (Vailhe 1998).

This work shows that producing a composite matrix by the admixture of collagen to fibrin, attenuates the migration of endothelial cells relative to a fibrin only matrix. This agrees with similar studies demonstrating that collagen type 1 retards tube formation by HMVEC cells seeded onto a fibrin gel in a dose dependent fashion (Kroon, van Schie et al. 2002). The addition of soluble angiogenic factors in this model showed a similar

attenuated response indicating possible opposing effects of converging intracellular pathways (Chapter 5.3).

The addition of other ECM components to collagen gels resulted in increased migration over collagen alone. Nevertheless, these proteins failed to stimulate to the degree of fibrin, although some matrix components stimulated greater endothelial cell invasive response than many growth factors/ cytokines used in the same assay (chapter 4).

Fibronectin has proven angiogenic properties. It is the only mammalian adhesion protein that binds to $\alpha_5\beta_1$ integrin (a proangiogenic integrin) (Vogel and Baneyx 2003) and also has sites for $\alpha_4\beta_1$, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ (induced in activated endothelial cells). Because fibronectin has binding sites for $\alpha_5\beta_1$, $\alpha_4\beta_1$ and $\alpha_v\beta_3$ and also has a collagen binding interaction a likely mechanism for its pro-angiogenic affects could be via increased engagement and signalling of multiple integrins on the endothelial cells. This is consistent with the present results. It also contains the anti angiogenic subunit Anastellin, a carboxy-terminal fragment of the first FN3 domain from human fibronectin, which has been shown to inhibit angiogenesis *in-vivo* (Yi 2003). Fibronectin contains extradomains B, which are markers of angiogenesis and remodelling, expressed in proangiogenic tissues such as solid tumours. These have been used as targets for anti tumour antibody therapy (Birchler, Milisavlijevic et al. 2003). Its polymerisation is critical in the regulation of ECM organisation and stability regulating cell proliferation, migration and differentiation (Sottile and Hocking 2002). Its role not only in angiogeniesis but also in vasculogenesis is essential in early blood vessel development along with its interaction with Integrin $\alpha_5\beta_1$ (Mao and Schwarzbauer 2005).

In this study, vitronectin increased endothelial cell migration in collagen gels by a factor of 1.6. No direct comparisons have been previously made between vitronectin and other matrices in their ability to impart an invasive phenotype in endothelial cells. Nevertheless, studies have confirmed its proangiogenic role in the wound environment and its potential to stimulate microvascular angiogenesis (Jang 2000). Adhering to SPARC, a glycoprotein, it modifies de-adhesion of cells from the extracellular matrix inducing cellular migration (Menon, Gutierrez et al. 2000). It mediates this action by ligating to vitronectin receptor type integrins (alpha$_v$ beta$_3$/ beta$_5$) and inducing an increase in endothelial cell proliferation and cell/extracellular matrix interaction inducing migration (Takagi 2002).

Both vitronectin and fibronectin are classic "RGD" matrix molecules. Vitronectin gave marginally greater migration than fibronectin yet the similarity of effect between fibronectin and vitronectin suggests that the RGD motifs, which both have, may underly this. This could be tested with RGD peptide competitive inhibitors of RGD integrin interactions. It is curious that migration in presence of fibronectin was modest in spite of the difference in integrin sites which might have been predicted to yield a stronger effect than vitronectin or fibrin. These results demonstrate that established "angiogenic" proteins may not provide the scaffold properties required for a SDE.

Gycosaminoglycans (GAGs) have been widely used in SDEs (eg chondroitin 6 sulphate in Integra (Yannas 1980) and heparan sulphate in Laser Skin (Lam 1999)). GAGs (chondroitin sulphate, dermatan sulphate, hyaluronan, heparan sulphate) are integral components of the ECM and basement membrane. They play an important role regulating immune and inflammatory responses by interactions with cytokines to control their presentation to cells and storage within the ECM (Fernandez-Botran, Gorantla et al. 2002). In the cytoball assay all the GAGs stimulated invasion, the greatest response was seen with chondroitin sulphate. This pro-angiogenic response is supported by other *in-vitro* studies, which concur that it induces a pro-angiogenic endothelial cell phenotype in matrigel matrices (Tapon-Bretaudiere 2002). Both hyaluronan and heparan sulphate induced more cell invasion than MM although this was relatively small. Other studies indicate the potential of hyaluronan as a potent proangiogneic GAG, stimulating angiogenesis within solid tumours (Franzmann 2003) and *in-vitro* chorioallantoic membrane angiogenic assays (Liu 2004). This may relate to the finding that hyaluronan oligosaccharides provide a strong proangiogenic stimulus yet the high molecular weight fractions are antiangiogenic (Lees, Fan et al. 1995). Leach used hyaluronic acid as the core protein within a cross-linked Synthetic Dermal Equivalent, concluding that it could serve as a biocompatiable hydrogel that provokes little inflammatory response and induces angiogenesis (Leach 2004), most especially at the graft periphery, to the same extent as a control fibrin gel. This angiogenic response contrasts with this chapter's results in which the hyaluronan, though slightly stimulating invasion in collagen, was not as great as VEGF (chapter 4) and less than a third that of fibrin. Heparan sulphate addition to collagen, in our assay, increased endothelial cell migration by a factor of 1.3 when compared to collagen alone. This corroborates with similar studies in which the addition of heparan sulphate to collagen biomatrices in an *in-vivo* setting increased

angiogenesis (Santhosh, Mathew et al. 1996). The marginal increase in angiogenic response from heparan may well be due to a deficiency of heparanases within the matrices. These have been shown to have potent pro-angiogneic effects (Vlodavsky, Elkin et al. 2000). Apart from their involvement in the egress of cells from the vasculature, heparanase acts both directly, by promoting invasion of endothelial cells (vascular sprouting), and indirectly by releasing heparan sulphate-bound basic fibroblast growth factor, and generating heparan sulphate degradation fragments that promote bFGF activity.

Laminin structurally, functionally and anatomically plays a clear role in regulating endothelial cell activity and migration. The present results show that laminin seems to have a low cell migratory effect although at low concentration does stimulate some migration. It's main effect is vasculogenesis and cell assembly rather than migration. It failed to support the same degree of invasion as that of fibrin and many others investigated. Significantly, when added to a fibrin/collagen matrix it profoundly attenuated endothelial cell migration. These results are broadly consistent with its effects promoting differentiation of HPMEC and assembly into capillary tubes.

Elastin, in the assay used in this chapter, stimulated angiogenesis when admixed in a collagen gel. Nevertheless such migration was small (1.6 times that of collagen) when compared to fibrin. Its addition to fibrin /collagen gels attenuated endothelial cell migration. Elastin mediates its effects by reorganising cellular actin filaments stimulating a change in cell shape. Direct comparisons between the pro-angiogneic effects of elastin and other ECMs have not been described. However it stimulated angiogenesis in the chorioallantoic membrane (CAM) model less than a synthetic polyurethane Tecoflex biopolymer (Zwadlo-Klarwasser 2001). Other groups have successfully used elastin as an artificial matrix or combined it with collagen. Elastin, with or without Lysine cross-linking, stimulated cell proliferation and angiogenesis (Girotti, Reguera et al. 2004).

The angiogenic action of Elastin has been attributed to elastin degradation proteins, most notably kappa elastin and VGVAPG hexapeptide elastin. These stimulated angiogenesis in the CAM assay as well as further stimulating endothelial cell migration and pseudo-tube formation in *in-vitro* matrigel and collagen matrices (Robinet 2005). VGVAPG injected into abdominal aortas in *in-vivo* studies stimulated a 100 fold increase in capillary density over controls (Nackman 1996).

Whilst the current data also identifies a pro-migratory response of elastin to endothelial cells its potential for incorporation into a second generation proangiogenic SDE is far less than fibrin. Again this data shows that the pro-angiogenic effect of elastin is unlikely to be useful as an SDE component.

The possibility that cell migration in an optimum mix of collagen and fibrin could be further stimulated by addition of a third ECM component was explored using 5 alternatives. It is notable that in all five triple matrices, migration was less than that of fibrin/collagen. The reason for this was not explored further but seems unlikely to be due to physical effects such as an increase in gel density or rigidity but more likely that ECM/cell interactions result in specific cellular responses.

One may hypothesise from the results that;

1) Both (a) motility (eg cytoskeletal activation) and (b) matrix remodelling (eg MMP secretion) may be stimulated by various growth factors and specific matrix interaction.

2) The balance between a and b influences the extent of invasion.

Some stimuli may result in MMP production or PA secretion causing matrix degradation to dominate over migration. This could be tested by using a broad spectrum MMP inhibitor such as Illomostat.

3.5 Conclusion

Angiogenesis is a complicated process or sequence of steps involving a change in endothelial cell phenotype from quiescence to motility, endothelial cell proliferation and tubulogenesis. This chapter has focussed on the potential of matrices to support the process of endothelial cell migration and infiltration because this must be critical for the take of synthetic matrices. It has established that, of those assessed, some ECM components, in the absence of growth factors, have greater impact on vascular endothelial cell invasion and hence angiogenic potential than others. Fibrin, as a primary component of wound healing, has greater influence on endothelial cell migration than collagen and is superior in this effect than any other component examined. This work identifies fibrin as a better proangiogenic scaffold than collagen for use in a future SDE.

This chapter represents the first step in defining a measurable criterion for selecting an ECM for inclusion in a SDE. Further chapters will investigate and compare the influence of growth factors/ cytokines as well as the mechanism of fibrin's effect and the influence of the macroscopic structure of the matrix on endothelial cell migration.

Chapter 4

A comparison of the activity of growth factors to stimulate endothelial cell invasion and angiogenesis. A therapeutic avenue to encourage the take of Synthetic Dermal Equivalents.

4.1 Introduction

Angiogenesis may be a critical factor in the healing of large wounds and is specifically important for the take of Synthetic Dermal Equivalents (SDEs).

Therapeutic angiogenesis relates to using pro-angiogenic factors to reverse the detrimental effects of ischaemia or optimise the vasculature of a tissue to maintain survival and function (Webster 2000). Promotion of vessel formation has progressed since the identification of angiogenic factors and their modes of action. The available modes of delivery (gene or protein) have generated avenues of therapy for wound healing either through direct injection or via the local circulation. Such therapy has been used with varied success in the field of Plastic Surgery in stimulating recalcitrant wounds, granulation tissue, maintaining skin flap viability and recently encouraging the take of Synthetic Dermal Equivalents.

This chapter aims to discuss the means of growth factor delivery, effects on growth factors on wound healing and investigate and compare the potential of various growth factors in stimulating endothelial cell invasion into a collagen matrix.

4.1.2 Background
4.1.2.1 Wounds and Cytokine profile

Following initial injury platelets accumulate within a wound. These cells initiate the cascade of growth factors present in the wound via release of PDGF, transforming growth factor beta (TGFβ) and EGF. PDGF is thought to direct the sequential migration of monocytes, neutrophils and fibroblasts to the site of wound repair. Activation and proliferation of early wound cells results in endogenous production of growth factors, which in turn act as chemo-attractants for other inflammatory cells to secrete regulatory growth factors or to migrate to the site of tissue repair to modulate the growth environment. Polymorphonuclear cells appear next and together with the arrival of macrophages predominate in the wound for the first two days following injury. Activated macrophages release a variety of factors into the wound, including IL-1 (Ford 1989). For this reason IL-1 levels detected in animal and human wounds have been observed to increase in concentration with the wound healing response (Grayson 1993; Vogt 1998). Fibrolasts, keratinocytes, endothelial cells and lymphocytes migrate into the site after the initial inflammatory reaction, providing a further cascade of growth factors, cell proliferation and extracellular matrix deposition (Kudlow, Kobrin et al. 1987).

Wound healing therefore requires a complex orchestration of these different cell types with regulation by growth factors and other cytokines. Various studies have measured endogenous levels of a variety of growth factors in healing wounds yet no single study has comprehensively analysed the profile of all major growth factors responsible for wound healing in a single wound. Studies observing the growth factors released from platelets PDGF, TGFβ and EGF show a consensus in that PDGF expression decreases with time to heal (Vogt 1998). In patients undergoing radical mastectomy the presence of PDGF related peptides within wound fluid was demonstrated confirming its presence within the healing wound environment (Matsuoka and Grotendorst 1989). However certain studies have shown that PDGF concentrations are lower than those within serum (Vogt 1998) while others have failed to detect PDGF (Grayson 1993). A similar concentration profile has been shown with both EGF (Vogt 1998) and TGFβ1 (Grayson 1993; Vogt 1998). This profile however is opposite to TGFβ2 which has been found to be in high concentrations in skin graft donor sites and increases in concentration with wound healing time. Elevated levels of PDGF and TGFβ have also been found in wound chambers (Grotendorst 1985) with TNF, IL-1 and IL6 and Macrophage Colony Stimulating Factor being shown to be secreted locally within a wound using a sponge matrix model (Ford 1989) implicating their roles in tissue repair. Despite such evidence implicating growth factor involvement in wound healing, many comparisons have been made between *in-vivo* studies and human wounds, varying in aetiology from incisional to skin graft donor sites, with conflicting results often complicating the situation and compromising understanding. The evidence as to whether any individual growth factor or sub group of factors has a predominant role in stimulating a healing response is therefore unclear.

4.1.2.2 Cytokine delivery, genetically modified dressings and chronic wounds

Successful acute wound healing relies on a balance between proteolytic activity, cell proliferation and matrix formation. Chronic wounds result from a disruption of the mediators (growth factors) of these activities (Falanga 1993) and an absence of matrix remodelling. Numerous studies have shown that chronic wound fluid has lower levels of growth factors such as PDGF, bFGF, EGF and TGFß than acute wounds (Cooper, Yu et al. 1994). Loss of growth factor activity by trapping of growth factors within the extracellular matrix, for example by glycosaminoglycan moieties, and degradation by proteinases, or reduced biosythesis has been proposed to explain chronic wound aetiology

(Hihley, Ksander et al. 1995). These findings have stimulated investigation of growth factor therapy for wound healing, promoting considerable interest over the last decade. Study results, though theoretically promising, have had mixed outcomes (table 4.1). This illustrated by the fact that to date only a single growth factor (PDGF as Regranex/Beclapermin) has been approved by the United States and European food and drug administrations for the treatment of chronic wounds.

Wounds have varied aetiologies with distinct growth factor requirements. If wounds are carefully chosen so that the processes affected by a given growth factor are the ones by which the wound progresses on a healing trajectory then exogenous application of recombinant growth factors may prove useful. The delivery of growth factors however must be considered. The modes and the vehicles for delivery are numerous, generally revolving around gene therapy and have crucial implications to wound outcome. This emphasised by Puolokainen who found that the ability of TGFβ to enhance wound healing depended on the carrier used for its topical delivery to the wound site (Puolakkainen, Twardzik et al. 1995).

Different delivery technologies for gene transfer have been investigated and applied to *ex-vivo* and *in-vivo* gene therapy in the field of wound healing and wound angiogenesis. The *ex-vivo* approach permits the introduction of genetic material directly into a particular cell type by isolating involved cells from the patient, genetic transfection of these cells in culture and autologous transplantion. Although limited by culture conditions and transplantation techniques, the strategy allows for control over target cell selection for genetic modification. This transfer technique has been used for keratinocyte culture methods producing genetically manipulated wound dressings. *In-vivo* gene therapy obviates the need for cell culture and transplantation since the genes are delivered directly to the target tissue. These methods require the DNA vector to be inserted into host cells *in-vivo*. Several viruses have been genetically engineered as "natural" vectors for *in-vivo* gene delivery. They have demonstrated the highest efficiencies for *in-vivo* gene transfer (Kozarsky and Wilson 1993; Naldini, Blomer et al. 1996). Adenoviruses and Vaccinia viruses can be concentrated to high titres but they pose significant viral cellular toxicity, stimulating inflammation and cellular destruction, and immune memory. Engineered Lentiviruses have several potential advantages, especially that their integration into the host genome is not dependent on cell proliferation. Although viral vectors are promising,

and their design is being refined, alternatives to *in-vivo* viral gene transfer are attractive in circumventing problems of tissue localisation and viral antigenicity.

Table 4.1 *In-vivo* and clinical evidence behind growth factor use in Plastic Surgery

Wound type	Study type	Outcome
PDGF		
Pressure Ulcers	a) 20 patients, randomised controlled, Regranex ™/ placebo over 29 days (Robson, Phillips et al. 1992) b) 124 patients, randomised, double blind, Beclapermin/placebo controlled, over 16 weeks (Rees, Robson et al. 1999) c) Double blind ,Placebo controlled Rh PDGF BB, 20 patients, 28 days – histological assessment (Pierce, Tarpley et al. 1994)	a) Regranex™/ placebo (94%-78%) reduction in wound volume b) ulcer volume reduction 93% beclapermin/ 73% placebo c) PDGF group – increased Fibroblast conc, new vessel formation, collagen deposition.
Diabetic Foot Ulcers	a) Multi centre, 118 patients topical Rh PDGF/placebo, 20 week trial (Steed 1995). b) Phase III randomised placebo controlled double blind study, 379 patients Beclapermin/Placebo, 20 week follow up (Wieman, Smiell et al. 1998). c) Multi centre, double blind, randomised Becaplermin/placebo controlled, 382 patients, 20 week trial (Wieman 1998)	a) 48/25% PDGF/Placebo completely healed (P= 0.01) b) 50/35% Beclapermin/placebo completely healed (P=0.007) c) 50/35% Becaplermin/placebo completely healed
Meta analysis		Beclapermin used once daily effective in improving diabetic ulcer care. Number needed to achieve healing in one extra patient varied 7-25 (Smiell, Wieman et al. 1999)
FGF		
Pressure Ulcers	Randomised placebo controlled, 50 patients, 30 days (Robson, Phillips et al. 1992)	69/59% FGF/Placebo reduction in wound volume (non significant result)
Diabetic ulcers	Randomised placebo controlled 17 patients 12 weeks (Richard, Parer-	33/63% FGF_2/placebo (non significant result)

	Richard et al. 1995)	
KGF **(animal studies)**		
Pig		Increased rate of epithelialisation in pig skin (Staiano-Coico, Krueger et al. 1993)
Rabbit		Epithelialisation promoted in ischaemia impaired rabbit ear wounds (Xia, Zhao et al. 1999)
EGF		
Pigs		Accelerates epidermal regeneration of partial/ full thickness pig wounds (Brown, Curtsinger et al. 1986)
Rat		Increased tensile strength in rat skin wounds (Brown, Curtsinger et al. 1988)
Venous ulcers	Randomised, placebo controlled, 10 week follow up (Falanga, Eaglstein et al. 1992)	35/11% EGF/Placebo complete healing (P=0.1) 73/33% EGF/Placebo reduction in ulcer size.
Diabetic ulcers	9 Patients, randomised placebo controlled cross over trial, 34 days (Brown, Curtsinger et al. 1991)	8/9 fully healed at 34 days
TGFβ		
Mice	Knock out mice (deficient in $TGF\beta_1$ (Crowe, Doetschman et al. 2000)	Delayed wound healing
Rabbit		Topical application of $TGF\beta_1$ fails to stimulate wound healing (Wu, Xia et al. 1999)
No human trials		
VEGF		
Rabbit		Topical VEGF improves granulation tissue formation in ischaemic wound healing (Corral, Siddiqui et al. 1999)
G-CSF		
Case report	Topical G-CSF in neutropenic patient with chronic wound (Cody, Funk et al.	Dramatic improvement in wound outcome

	1999)	
Diabetic foot ulcers	Randomised, placebo controlled, 40 patients (Gough, Clapperton et al. 1997)	20/0% GCSF/Placebo fully healed at 7 days (P= 0.09)

Non-viral *in-vivo* gene transfer techniques are particularly attractive for topical delivery and include direct injection (Hengge, Chan et al. 1995). A more sophisticated technique of puncture mediated gene transfer or micro-seeding for delivery of naked DNA has recently been described by Ciernik (Ciernik, Krayenbuhl et al. 1996). This technique delivers DNA to target cells by multiple needle perforators. These models used EGF and detection efficacy by studying target gene expression in tissue and wound fluid at transfected wound sites. The authors concluded that micro-seeding is more efficient than single dermal injection. A further type of physical method for introducing DNA into cells is by particle mediated gene transfer (gene gun). This uses DNA coated particles or micro projectiles accelerated by force i.e. high voltage (Baragi, Renkiewicz et al. 1995), electrical discharge or high-pressure Helium (Klein and Fitzpatrick-McElligott 1993). This method remains limited by poor stable integration and transient gene expression but has met some success in animal models. Cationic liposomes coated with DNA provide an alternative non-viral gene transfer technique. Liposome-DNA non-covalently bonded complex is added to target cells, adsorbed into the cell membrane and delivers the DNA to the cytoplasm (Li and Hoffman 1995). This method takes advantage of the anionic DNA properties, cationic liposomes and the negatively charged cell surface. Liposome vectors provide few constraints on gene size for delivery and lack toxicity. This aside, "lipofection" is associated with a low efficiency of transfection (Krishnamoorthy, Morris et al. 2001).

Despite encouraging growth factor studies (table 4.1) a limitation of this approach is that most trials are confined to single growth factors influencing specific wound types. This is a general criticism of single agent therapy not specifically of these trials as such. It is however unlikely that one remedy or growth factor will avail all eventualities. Clearly individual growth factors should be targeted at those components or processes that a given wound uses to heal. For example, partial thickness wounds heal entirely by epithelialisation. These can respond to KGF/EGF treatment (table 4.1). By contrast, deep chronic pressure ulcers require little epithelialisation and heal by contraction, angiogenesis, fibroplasia and matrix synthesis and respond to TGFβ and PDGF therapy. Steroid induced wounds are a distinct type, which fail due to a lack of inflammation.

Their healing can be restored by administration of Vitamin A (stimulating monocyte differentiation) or multiple growth factors (Ksander, Chu et al. 1990). Sequential and combination therapy may provide the basis of future therapies and provide an answer to wound healing indolence (Robson 1991) yet the clinician needs to be able to fully understand the wound aetiology and pathology to give a growth factor solution, as well as use a relevant carrier as eluded to earlier.

4.1.3 Synthetic Dermal Equivalents (SDE) and growth factors

Human skin equivalents that contain cells, principally keratinocytes and or fibroblasts, naturally release growth factors such as VEGF (Casasco 2001) which stimulate endogenous cells. The production of multiple stimulatory factors from such cells is very much dependent on cellular interaction with the ECM.

While cellular action can be stimulated by the ECM certain studies have investigated the effect of incorporated growth factors within the SDE on cellular motility. Gosiewska showed that PDGF stimulates fibroblast migration into SDEs (as well as stimulating wound healing and tissue repair) (Gosiewska 2001) which in turn remodels the SDE (Lee 2000). SDEs cultured with TGFβ_1 *in-vitro* not only stimulated a proliferation of fibroblasts but a 6 fold increase in collagen production (Bell 1991; Jutley, Wood et al. 1993). The same applies for IL-4, when added *in-vitro* to a collagen SDE, the growth factor increases collagen synthesis in a concentration dependent manner from stimulated migrating fibroblasts (Fertin 1991). Incorporating growth factors such as TGFβ_1 in a collagen lattice however has been shown to not only stimulate fibroblast activity but induce a fibrotic response increasing dermal contraction and protein synthesis such as fibronectin, tenascin and smooth muscle actin (Gentilhomme 1999). Less research has investigated the effect of growth factors on endothelial cell migration into SDEs, a key process in the take and survival of such bioengineered skin replacements (Converse 1975). In one study on the effects of FGF and VEGF$_{(165)}$ on endothelial and fibroblast phenotype within a heparin carrying polystyrene bound collagen dermal replacement, these growth factors significantly increased trans-matrix endothelial cell invasion (Ishihara 2001).

Clinical studies have thus shown that growth factors have a role in the wound healing process and have been used via gene therapy to improve wound healing. Whilst having a direct effect on angiogenesis, little has been published on the optimum growth factor for

the stimulation of angiogenesis within the wound and into SDEs. Different growth factors and cytokines have been studied *in-vivo* and *in-vitro* but the specific direct effects on endothelial cells vary depending on the system. In using a human microvascular endothelial cell *in-vitro* model, this chapter aims to quantitatively study cellular invasion in direct response to the pleiotrophic effects of agents such as PDGF, VEGF, TNFα and IL-1β with the more endothelial specific agents such as VEGF, aFGF and bFGF as well as chemokines known to act on endothelial cells such as IL-8. This identification of an optimum growth factor for potential use in promoting endothelial cell invasion into skin replacements has great clinical implications.

4.2 Material and Methods

4.2.1 Cell Culture

Endothelial cells were cultured *in-vitro* using M199 with Earle's salts supplemented. Angiogenic stimulants were prepared as outlined earlier (chapter 2.2.9) each at three concentrations.

4.2.2 Cytodex bead assay

The cytoball assay (chapter 2.2.4) was used to quantify the degree of endothelial cell migration. Cytodex beads™ (Pharmacia Biotech) were swollen and prepared from dehydrated stocks.

Twenty-four-well plates were pre-coated with 100µl of sterile 1% (LMP) agarose gel (Sigma) per well. 100 µl of cytodex sphere suspension (approximately 250 spheres), 750,000 endothelial HPMEC cells and 500 µl of minimal media were added per well. The cells and beads were incubated at 37°C for 24 hrs to establish confluent bead cell cover.

Endothelial cell migration assays were established by suspending confluent beads in collagen gels (0.75mg/ml). This was shown to provide an optimal gel density for migration (chapter 3.3.1). Approximately thirty beads were aspirated from the confluent bead cultures mixed in a sterile universal container with the ECM and gently pipetted into the wells. All experiments used at least three cell lines. Once gelled (30 minutes), equal volumes of media with or without angiogenic stimulants were added to the matrix.

The media and gel were incubated at 37 °C with 5% CO_2 for 96 hrs, all media and stimulants were exchanged at 48 hrs and the gels fixed (4% Paraformaldehyde) at 96 hrs. This assay allowed comparisons of soluble angiogenic proteins for endothelial cell invasion. The angiogenic proteins were prepared at specific concentrations in minimal media.

4.2.3 Migration assay

Cytodex beads were photographed and an average cell invasion distance from each bead was calculated using Sigma Scan Pro image measurement software™. For each bead a mean migratory distance was established for ten cells (chapter 2.2.4). For each condition 30 beads and three hundred cells were evaluated. For each condition a mean migratory

distance (microns) and standard error of the mean was established from the thirty beads. This process was repeated with the different cell lines.

Data was normalised to the control migration in MM in order to combine data from different cell lines.

4.2.4 Statistical Analysis

ANOVA analyses of pooled (raw) migration data was performed using the Sigma Stat 2 statistical software package. Differences between groups were tested with Tukey-Kramer post hoc test and were considered significant if the p value was less than 0.05.

4.2.5 Soluble angiogenic factor stimulants

Angiogenic stimulants were prepared from stocks each at three different concentrations using minimal media (table 4.2).

Each stimulant was prepared at three concentrations and added in equal quantities to the gelled collagen. 5% serum was used as a positive control and was prepared by the addition of sterile irradiated foetal calf serum to minimal media.

4.3 Results

As expected, invasion was relatively low in the MM negative control and maximum in the serum positive control. Migration of endothelial cells with the addition of the angiogenic stimulants in MM gave values between the positive and negative controls (Tukey, $p < 0.05$, table 4.2).

Angiogenic stimulant	Concentrations used	Migration relative to MM	Migration greater than MM (microns)
Serum	5%	1.99	70.86 +/- 44
bFGF	3,10,30 ng/ml	1.56	53.25 +/- 26.75
ECGS	37.5, 75, 150µg/ml	1.70	35.5 +/- 23
IL-1ß	10,30,100 ng/ml	1.29	29.5 +/- 15.5
IL-8	2.5,10,25 ng/ml	1.45	44.75 +/- 30
PDGF	1,3,10 ng/ml	1.72	34.7 +/- 12.3
TGFβ$_1$	0.5, 1.3, 5 ng/ml	1.98	69.13 +/- 42.8
TNFα	20, 100, 500 U/ml	1.27	22.8 +/- 28.2
TPA	0.05, 0.5 5 ng/ml	1.15	2 +/- 32
VEGF-A	0.5, 5, 50 ng/ml	1.82	45.5 +/- 14.4

Table 4.2

Relative migration and migration in microns given by the optimum concentration of each growth factor investigated.

bFGF

Addition of bFGF to endothelial cells in collagen gels at all concentrations studied gave greater migration than the negative control (MM) ($p < 0.05$, n =4, Tukey), There was no statistical difference between 10 and 30ng/ml nevertheless greatest migration was seen with 10ng/ml, giving 1.56 times the migration of MM.
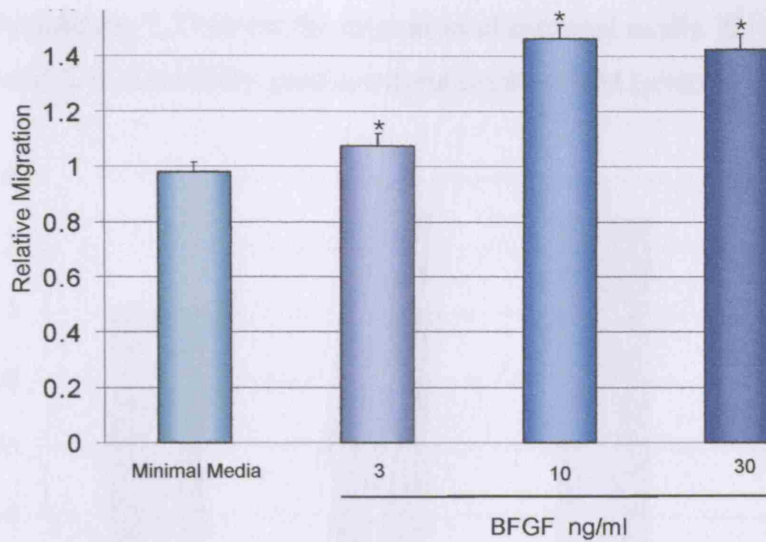
Figure 4.1 Endothelial cell invasion into collagen gels with or without the addition of bFGF to minimal serum media (mn +/- SEM, * p < 0.05, n=4)

ECGS

The addition of ECGS to endothelial migration in collagen gels was concentration dependent. Maximum migration was seen with 150µg/ml giving 1.7 times the migration of MM. All three concentrations stimulated a statistically greater increase in migration than MM (p<0.05, n=4 Tukey test)
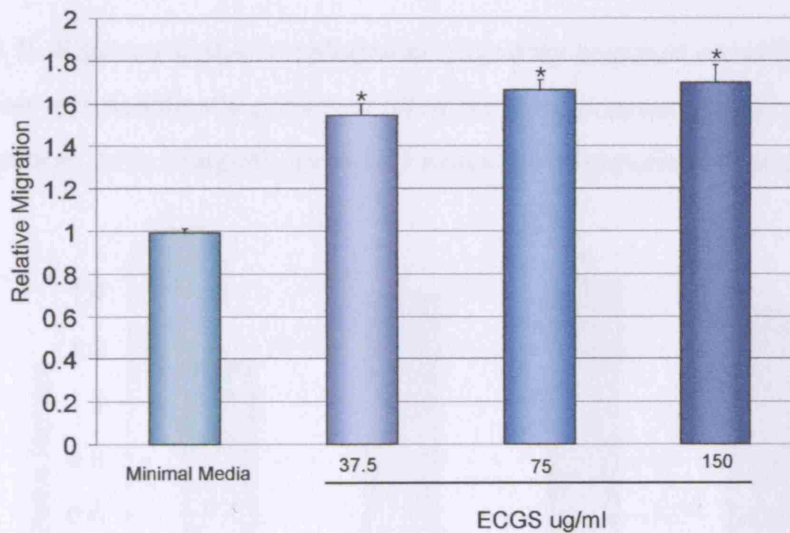
Figure 4.2 Endothelial cell migration in collagen gels was proportional to the concentration of ECGS added (mn +/- SEM, * p < 0.05, n=4).

IL-1ß

Addition of IL-1ß to collagen matrices seeded with endothelial cells induced endothelial cell migration inversely proportional to IL-1ß concentration. Maximum migration was

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with 10 ng/ml producing 1.29 times the migration of minimal media. IL-1ß at all three doses used provided a statistically greater migration than MM (p<0.05, n=4, Tukey test)
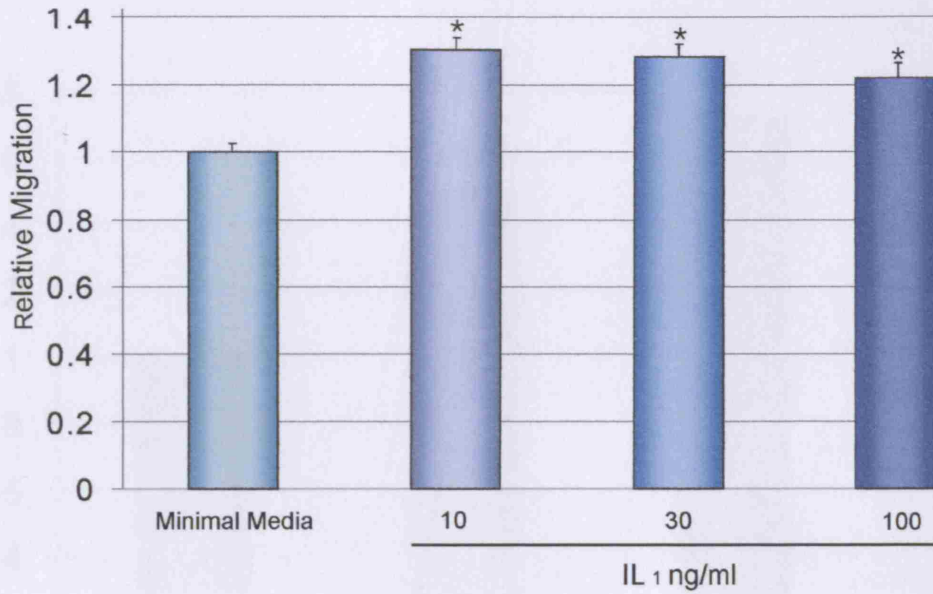
Figure 4.3 Endothelial cell migration was inversely proportional to IL-1ß concentration in collagen gels (mn +/- SEM, * p < 0.05, n=4).

IL-8

Unlike IL-1ß, IL-8 gave a biphasic endothelial migratory response on addition to Collagen gels. Migration was statistically greater at all of the three concentrations tested over MM. Greatest migration, with 10 ng/ml, gave 1.45 times the migration of MM alone.
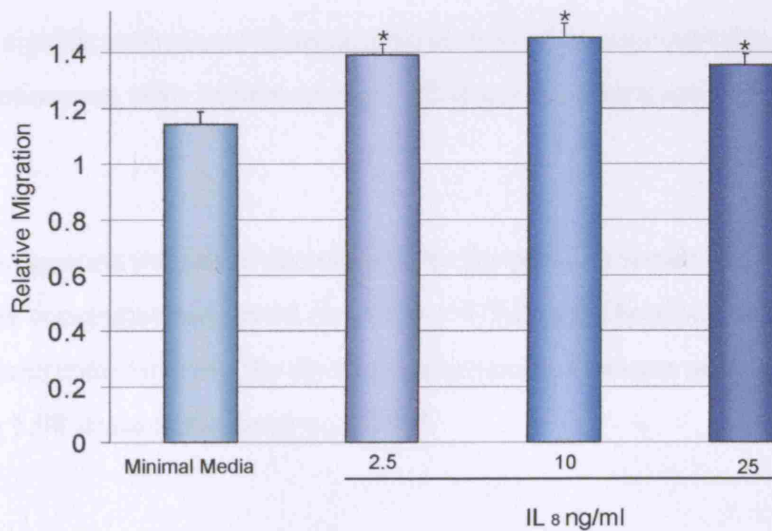
Figure 4.4 Migration of endothelial cells in collagen gels gave an increase in migration with the addition of IL-8 compared to minimal media (mn +/- SEM, * p < 0.05, n=4).

PDGF

PDGF gave a statistically significant increase in migration with the lower concentrations 1 and 3 ng/ml, when compared to MM (p<0.05, n=4, Tukey test).
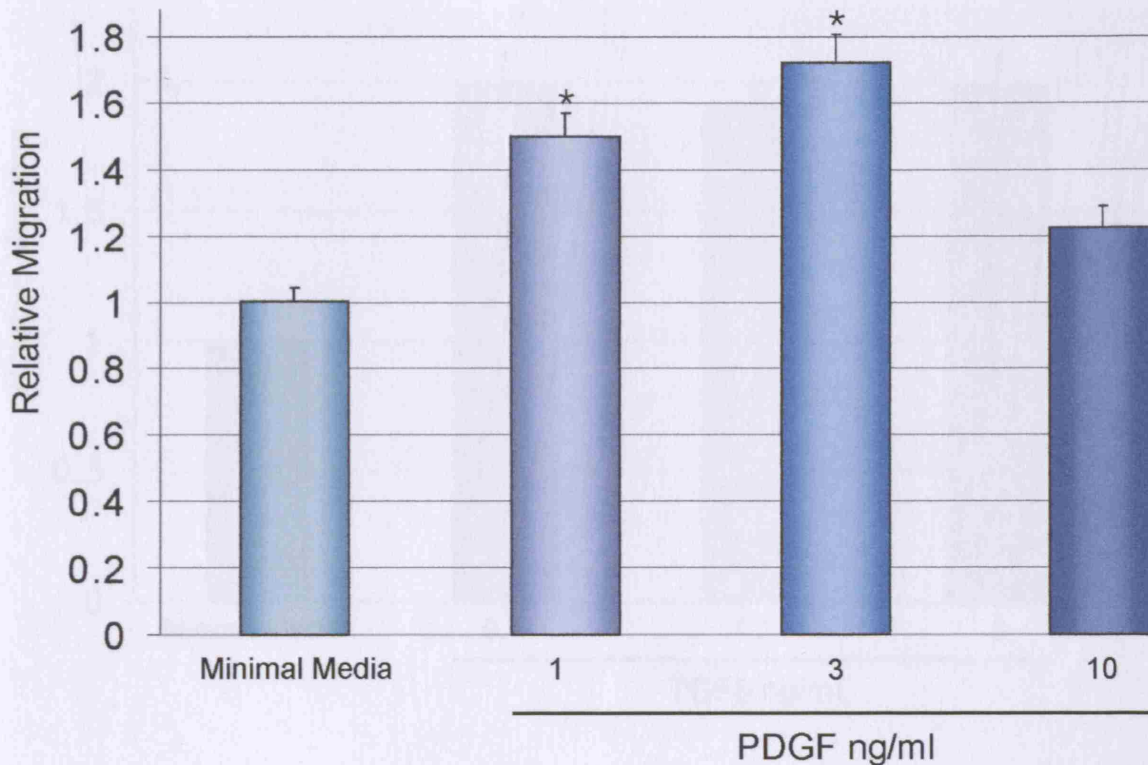
Figure 4.5 The biphasic endothelial cell migration response seen with the addition of PDGF to collagen gels. Only the lower concentrations gave a significant increase in migration (mn +/- SEM, * p < 0.05, n=4).

There was no significant increase in migration at the higher concentration 10 ng/ml. Greatest migration was with 3ng/ml giving 1.72 times the migration of MM.

TGFß-1

TGFß gave the greatest migration second only to the positive serum control. This was the case at all three concentrations tested (p<0.05, n=4 Tukey). There was no statistical difference in migration between the three concentrations. Greatest migration was with 5ng/ml giving 1.98 times the migration of MM.
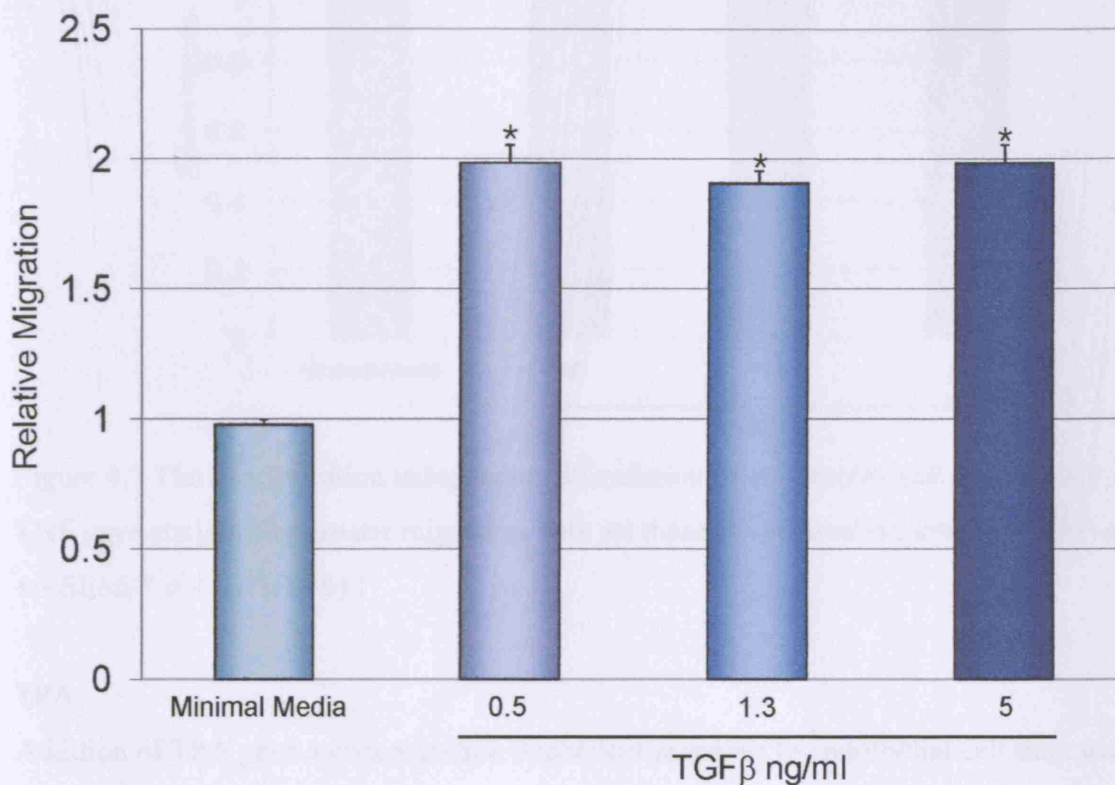
Figure 4.6 TGFß stimulated greater endothelial cell migration than the other growth factors. Greatest migration was with 0.5 ng/ml. All three concentrations assessed increased migration by a factor of nearly 2 over the migration of MM (mn +/- SEM, * p < 0.05, n=4).

There was no statistical difference in migration seen with 5% serum (1.99) and TGFβ 5ng/ml, proving that TGFβ was as effective as whole serum (p>0.05, Tukey).

TNFα

TNF gave a concentration independent increase in migration over MM, over the range studied. Maximum migration found was from 20 U/ml giving an increase in migration of 1.27 compared to the minimal serum control (22.8 +/- 28.2 microns). All three concentrations of TNF gave a statistically significant increase in migration. Nevertheless there was no difference statistically between the concentrations (p< 0.05, Tukey, n=6).
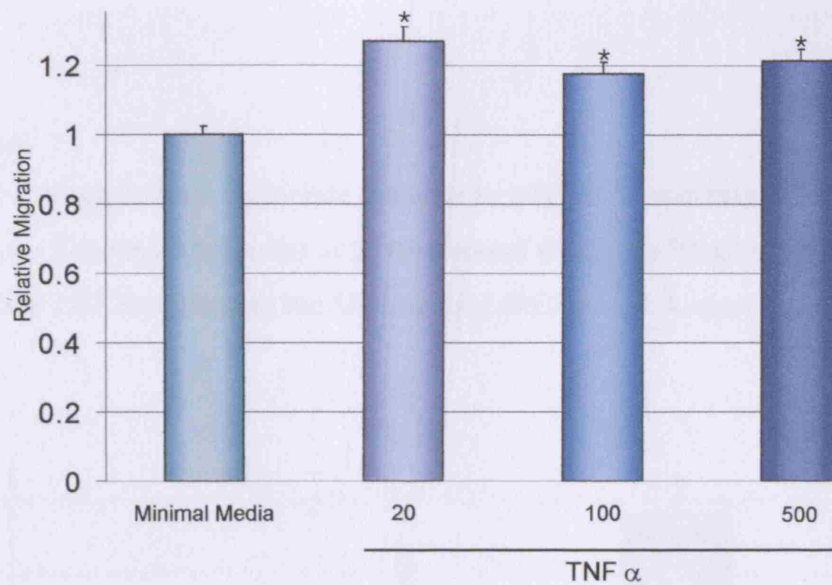
Figure 4.7 The concentration independent stimulation of endothelial cell migration with TNF gave statistically greater migration with all three concentrations tested over MM (mn +/- SEM, * p < 0.05, n=6).

TPA

Addition of TPA gave a concentration dependent response to endothelial cell migration over the range studied. A small increase in migration was only seen at the highest concentration tested (5ng/ml, p< 0.05, n=3). This gave 1.15 times the migration of endothelial cells with a minimal medium.
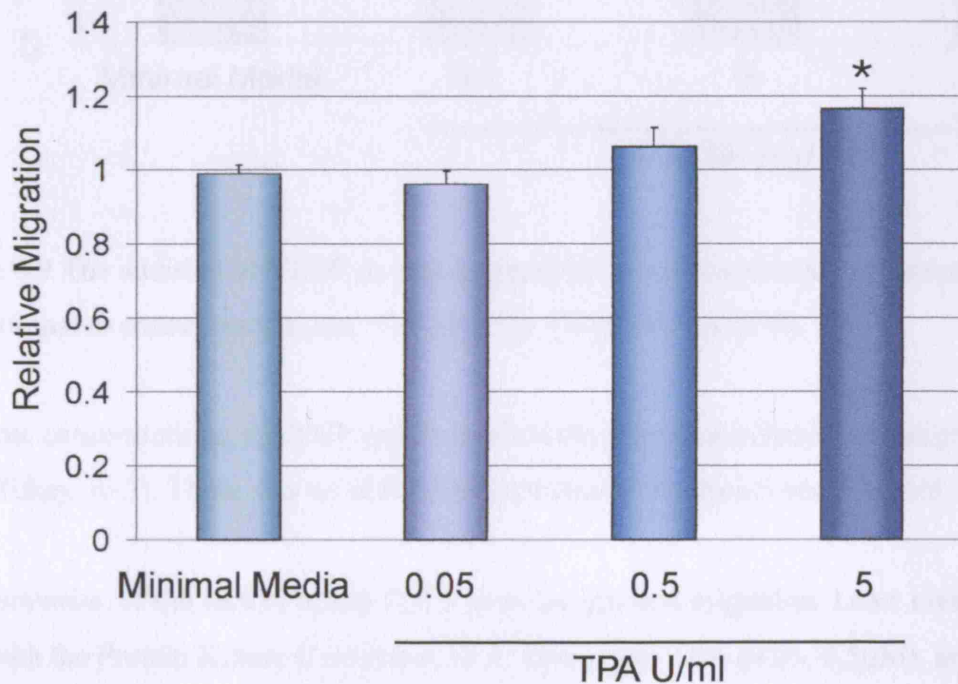
Figure 4.8 TPA gave a statistical increase in endothelial cell migration over minimal medium only at the highest concentration tested (mn +/- SEM, * p < 0.05, n=3).

VEGF-A [165]

VEGF gave a concentration dependent increase in migration over minimal medium, over the range 0.5 – 50ng/ml. Maximum migration found was from 50ng/ml giving an increase in migration of 1.82 compared to the MM control (45.5 +/- 14.4 microns).
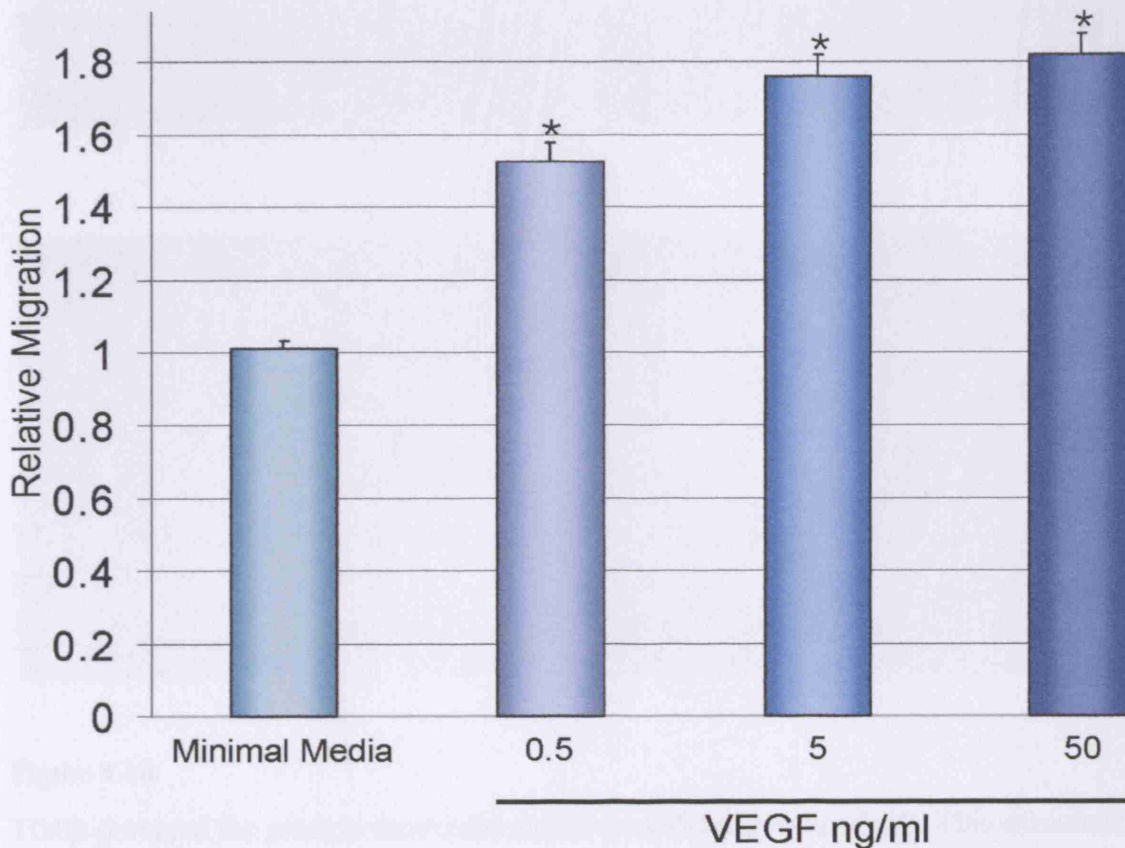
Figure 4.9 The addition of VEGF to collagen gels increased endothelial cell migration proportional to concentration (mn +/- SEM, * $p < 0.05$, Tukey, n=7).

All three concentrations of VEGF gave a statistically significant increase in migration ($p < 0.05$, Tukey, n=7). There was no difference statistically between 5 and 50ng/ml ($p > 0.05$).

To summarise, of the factors tested TGFβ gave the greatest migration. Least invasion was seen with the Protein Kinase C activator TPA. Other than TPA (0.05, 0.5µM), angiogenic stimulants stimulated invasion across all concentrations tested.
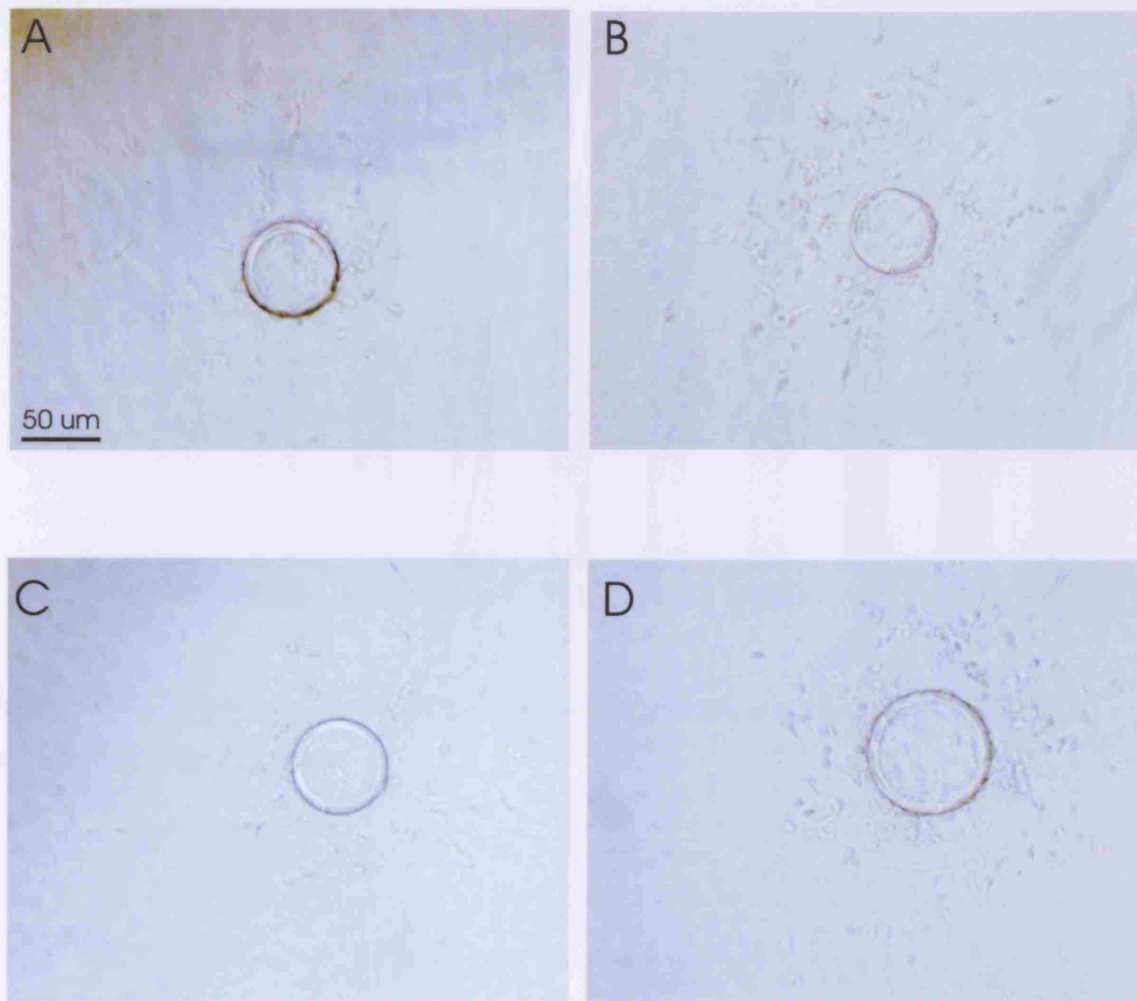
Figure 4.10

TGFβ provided the greatest stimulation of all growth factors tested (B). This stimulation was greater than that of IL-1, and TNF (A and C). bFGF (D) provided less stimulation than IL-1 and TNF, it contrasted significantly ($p < 0.001$, ANOVA) in invasive response to TGFβ.

PDGF, bFGF and IL-8 stimulated invasion in a biphasic concentration dependent manner with ECGS, IL-1, TGFβ and TNF stimulating invasion independent of concentration (Table 4.2, Figure 4.10)
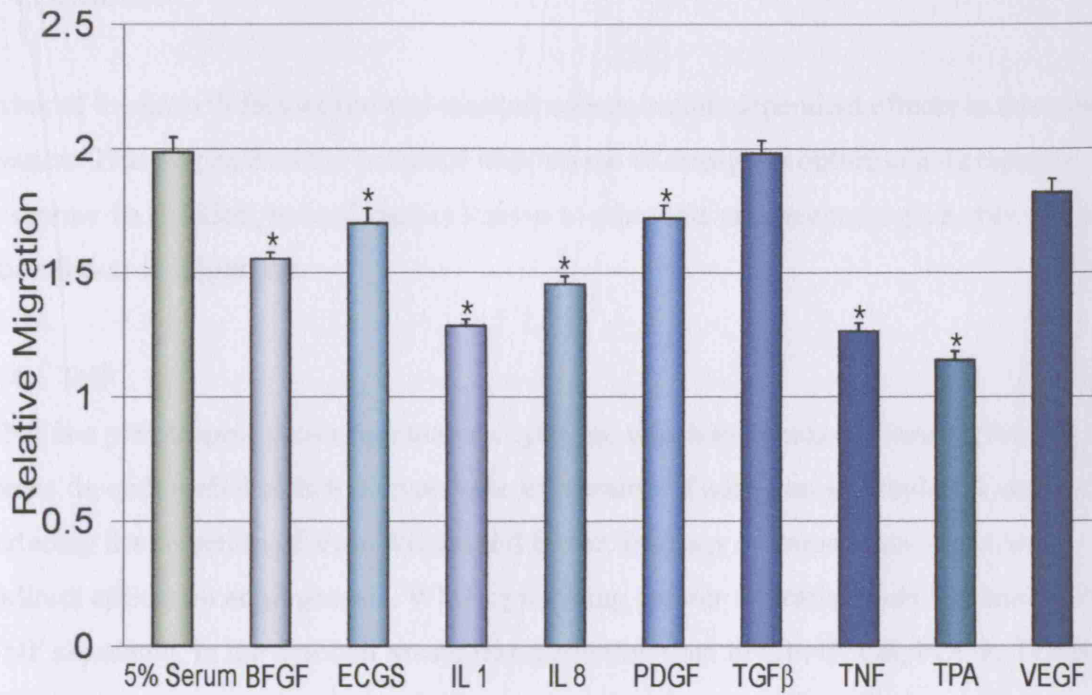
Figure 4.11 Comparison of endothelial migration from the optimum concentrations of each growth factor assessed.

4.4 Discussion

Most of the growth factors showed marked concentration dependent effects in this assay system. This emphasises the potential importance of dosage to optimise a therapeutic response. In addition, several factors known to stimulate angiogenesis gave only modest stimulation of migration.

4.4.1 TNF

TNF is a pleiotrophic pro-inflammatory cytokine which mediates activation of many genes. In endothelial cells it activates the expression of adhesion molecules as well as inducing the secretion of Von-Willebrand factor. In many systems it has direct and indirect effects on angiogenesis. Whilst providing greater migration than minimal media TNF simulated, in the cytoball assay, less migration than bFGF, ECGS, PDGF, TGFβ and VEGF. There was no significant difference in migration from IL-1, IL-8 and TPA ($p > 0.05$). Similar *in-vitro* studies have compared endothelial cell migration in collagen gels with/out TNF and have found corroborating stimulation of proliferation and migration (Cai and Liu 1981). Szekanecz (Szekanecz, Shah et al. 1994) further showed that anti TNFα antibody significantly inhibited endothelial cell chemotaxis ($p < 0.05$) suggesting a role for TNF in neovascularisation. This chapter has shown a promigratory effect of TNFα, yet this was mild compared to other soluble angiogenic factors. This contrasts with other *in-vitro* and *in-vivo* studies in which TNFα is found to have similar migratory/angiogenic effect to bFGF (Vanderslice, Munsch et al. 1998) and VEGF. TNFα is well known to induce pleiotrophic effects on neutrophil function (Ferrante, Kowanko et al. 1992) as well as endothelial cell phenotype and angiogenesis. It plays an important role in the wound healing response stimulating endothelial cells to express vascular cell adhesion molecule (VECAM) acting on $\alpha_4\beta_1$ integrins (Vanderslice, Munsch et al. 1998) to mediate its angiogenic effects. While we have measured only a mild direct pro-migratory effect on endothelial cells, it's effects on tubulogenesis and cell proliferation, not observed in this system, may account for the potent angiogenic effects observed in other *in-vivo* studies.

4.4.2 TGFβ₁

TGFβ induced the greatest invasive response of all factors tested, as much as 5% serum (figure 4.10). No comparable study has observed endothelial cell invasion in collagen gels in response to TGFβ with comparisons to alternative growth factors, although its pro-

angiogenic effects have been studied. Bell noted that at concentrations of 20ng/ml, TGFβ₁ reduced bovine endothelial cell proliferation and migration *in-vitro* compared to control conditions (Bell 1991), this being less than PDGF, Histamine, Nor-epinephrine and phenoxybenzamine. Similar anti-angiogenic effects were noted by Passaniti (Passaniti 1992) in which TGFβ₁ inhibited the neo-vascularisation of matrigel previously stimulated by bFGF *in-vivo*. Many studies nevertheless indicate TGFβ₁ as pro-angiogenic noting a dose dependent increase in migration of *in-vitro* bovine endothelial cells (0.3- 0.7 ng/ml) (Gajdusek, Luo et al. 1993) however higher doses, unlike our study, were thought to be inhibitory. Similar dose responses were likewise noted to stimulate aortic and small vessel bovine cells to proliferate (Iruela-Arispe and Sage 1993). Other studies have noted that at concentrations greater than 5 ng/ml, TGFβ₁ induces an inhibitory effect (Pepper, Vassalli et al. 1993).

TGFβ₁, like bFGF, may induce the angiogenic effects by controlling the proteolytic environment of the ECM (Iruela-Arispe and Sage 1993). It has been found to increase PAI and decrease UPA mRNA synthesis producing an anti-proteolytic environment (Pepper, Belin et al. 1990).

TGFβ₁ is a pleiotrophic factor with a critical function for wound healing. However its direct effects on endothelial cells appear to be diverse and in some experimental systems it has inhibitory actions on endothelial cell penetrance and angiogenic behaviour but in other systems it is stimulatory and depends on the receptor type I isoforms (ALK5, ALK1) induced.

4.4.3 TPA

TPA or PMA belongs to a class of naturally occurring compounds called phorbol esters, which activate multiple Protein Kinase C isoforms and hence down stream proliferative pathways in cells mimicking, *in-vitro,* a number of activation events occurring during inflammation. Work by Montesano found a stimulatory effect of TPA in both collagen (Montesano 1985) and Fibrin (Montesano 1987) gels. These *in-vitro* studies investigated endothelial cell migration through a dense gel from an overlying cultured monolayer. Other groups have used a system similar to the cytoball assay seeding human umbilical vein endothelial cells (HUVEC) on gelatin beads with investigation of subsequent migration into fibrin. Such assays have found that TPA, used at similar concentrations to

the cytoball experiment, with concomitant addition of bFGF and VEGF, stimulated migration to nearly twice that of control conditions.

HPMEC exposed to collagen in MM show a "sprouting" pattern of response. It would be interesting to find out whether TPA might be a significant stimulus to drive cells into a migratory response. This data indicates that Protein Kinase C stimulation is not sufficient to drive migration.

4.4.4 bFGF/ECGS

Fibroblast growth factors were originally described as a mitogen for mesenchymal cells but have since been established as potent angiogenesis factors with other wound healing functions. The FGF family consists of at least seven members including both acidic and basic forms which have 50% homology in their amino acid sequence. aFGF and bFGF are released by cells through poorly understood mechanisms and exert their effects in stimulating collagen synthesis, wound contraction, epithelialisation, matrix synthesis and angiogenesis.

In general the activity profiles of aFGF and bFGF are difficult to distinguish, although aFGF and ECGS are heparin dependent

As has been shown in other collagen invasion assays (Vernon and Sage 1999) we found that bFGF stimulated a biphasic concentration dependent effect on endothelial cell migration (figure 4.1). The optimum concentration of bFGF, 10 ng/ml in this study, concurs with other studies investigating the angiogenic effect on aortic endothelial cells in 3D collagen gels. Gajdusek found that 0.5ng/ml was the optimum concentration for a morphological change in endothelial cells yet migration was most effective at concentrations greater than 2.5ng/ml (Gajdusek, Luo et al. 1993). ECGS was somewhat more potent.

In the cytoball study, bFGF stimulated greater migration than TPA, TNF, IL-8, IL-1, yet less than VEGF, TGFβ₁, PDGF ECGS (figure 4.10). This contrasts with similar studies in which bFGF in a basement matrix gel was more potent in stimulating Human Umbilical Vein Endothelial Cells (HUVEC) to migrate than VEGF, PDGF, ECGS, IL-8 and HGF (Kumar 1998). However these studies differ in cell origin and extracellular matrix.

The chemotactic effect of bFGF for endothelial cells through collagen gels in this system, though refuted by certain studies (Szekanecz 1994), corroborates numerous other studies (Nehls, Herrmann et al. 1998; Nakashio, Fujita et al. 2002). Certain studies have reported that bFGF has no chemotactic role on endothelial cells (Szekanecz 1994). Others have reported that it requires other factors to induce cellular migration, especially when compared to IGF-1, VEGF or PDGF. However when added to these factors, four fold increases in migration occur (Castellon 2002). bFGF is thought to induce cellular migration by altering the proteolytic activity of the endothelial cell (Pepper, Mandriota et al. 1998) through secretion of plasminogen activators (Pepper, Sappino et al. 1992), collagenases and Matrix Metalloproteinases (Passaniti 1992) that allow endothelial cell invasion *in-vitro*.

There are four FGF receptors which are thought to transduce FGF stimulation but it is not clear whether there is a relation between which receptors are expressed in particular endothelial cells and relative response to aFGF (ECGS) or bFGF. Their system presents a good opportunity to investigate this question experimentally.

4.4.5 IL-1β

IL-1 historically is known as a pleiotrophic pro-inflammatory cytokine with effects on neutrophils with a similar profile to TNF. It stimulates neutrophil cellular migration, respiratory burst, cell adhesion and cell surface receptor expression (Ferrante 1992). It is known to stimulate endothelial to neutrophil cellular interaction and alter endothelial cell adhesion increasing neutrophil transmigration (Takahashi 1995) and could potentially influence endothelial migration. This change in cell behaviour could well account for the migration observed in our study.

IL-1 however, only stimulated endothelial cell invasion over the concentration range studied (figure 4.3), being less than all factors tested except TNF and TPA. An *in-vivo* study using a matrigel basement membrane gel inserted in a skin chamber in mice found contrasting evidence that IL-1 injection into the matrices inhibited neo-vascularisation (Passaniti 1992). This *in-vivo* data supports *in-vitro* human umbilical vein endothelial cell studies showing no change in migration or proliferative phenotype with IL-1ß antibodies.

4.4.6 IL-8

IL-8 is a chemokine produced by phagocytes and mesenchyme exposed to inflammatory stimuli it activates neutrophils inducing chemotaxis, exocytosis and the respiratory burst and is known to activate endothelial cells in some systems. When injected, it induces neutrophil accumulation (Baggiolini, Imboden et al. 1992). We found that IL-8 induced a biphasic endothelial invasive response at concentrations of 2.5 to 25 ng/ml (figure 4.4). The optimum concentration was 10 ng/ml, this stimulated a greater endothelial invasive response than IL-1, TNF and TPA (figure 4.10). This validates other data which shows that IL-8 is a potent stimulus for endothelial cell invasion (Nickoloff 1994) and chemotaxis (Szekanecz 1994). The finding in this study that IL-8 induced less migration than TGFβ, VEGF, PDGF, ECGS and bFGF relates well with Kumar's study showing that IL-8 produces less migration than HGF, bFGF and VEGF-A and dramatically less mitogenesis than bFGF, VEGF and PD ECGF (Kumar 1998). Although it may have a therapeutic role in stimulating circulating endothelial cells, its use to stimulate graft angiogenesis does not seem warranted.

4.4.7 PDGF

PDGF is also pleiotrophic and an important growth factor in serum. PDGF is thought to have a role in capillary maturation but effects on initial EC migration are less well studied with some contradicting evidence.

Platelet derived growth factor, released from the α-granules of the platelet is a 30-32 KD glycoprotein with two subunits, A and B, which share 56% homology. While the AB hetrodimer is the primary product of human platelets, the effects of PDGF BB on endothelial cell invasion were chosen for study owing to its license for chronic wound treatment. PDGF AA, investigated *in-vitro* has previously failed to stimulate an invasive/migratory response (Thommen, Humar et al. 1997).

PDGF exerted a dose biphasic dependent effect on endothelial cell invasion with a maximum response seen at 3 ng/ml. Iguchi similarly showed in a rabbit *in-vitro* model that at high concentrations (10-30 ng/ml) PDGF BB was inhibitory to endothelial cell migration and that the optimal concentration was 3-10 ng/ml. Phillips similarly showed that endothelial cell migration was dependent on PDGF concentration (0.1 – 3.0 ng/ml) (Phillips and Stone 1994). Others have found higher doses (100ng/ml) to be pro-migratory to aortic endothelial cells, increasing migration by 67% over controls

(Thommen, Humar et al. 1997). Our study showed that PDGF stimulated endothelial cell migration greater than bFGF, TNF, TPA, IL-1 and IL-8 (figure 4.10), comparative studies have shown that PDGF stimulates a 2.5 fold increase in migration than controls, this being greater than VEGF, ILGF, bFGF and PLGF (Castellon 2002). A biphasic *in-vitro* response suggests that dosage could be critical for *in-vivo* responses and that high uncontrolled dosage could be clinically counterproductive.

4.4.8 VEGF-A [165]

VEGF is an endothelial specific cytokine that is widely expressed in rapidly growing and injured tissues such as tumours and healing wounds (Karayiannakis 2003). It acts through receptors expressed on vascular endothelium, $VEGFR_1$ and $VEGFR_2$. It increases microvascular permeability, re-programmes gene expression, promotes endothelial cell survival, prevents senescence, induces endothelial cell division (Nakashio, Fujita et al. 2002) and, as shown in this study, stimulates endothelial cell migration.

VEGF-A is recognised as an archetypal angiogenic stimulant. It has several splice variants with differing heparin binding affinities as well as different matrix cell surface sequestration. The concentration dependent effect of VEGF in this study was similar to Vernon's 3D *in-vitro* endothelial cell invasive model (Vernon and Sage 1999). Similar *in-vitro* studies have further shown the potent angiogenic pro-migratory effect of VEGF. Using an *in-vitro* assay of endothelial cell invasion/ migration, Kumar showed that, of 6 cytokines tested, VEGF gave a very potent invasive response, surpassed only by bFGF, and was found to be the only cytokine to increase trans-endothelial cell monolayer permeability (Kumar 1998). Similar comparisons of cytokines in an *in-vitro* migration assay on bovine retinal endothelial cells have shown that VEGF provides a two-fold increase in migration, this greater than PDGF, IGF, bFGF and PLGF (a $VEGF_{R1}$ specific agonist). Though other studies concur with this data, 3D invasion assays have found that VEGF yields a greater invasive response than $TGF\beta_1$ (Vernon and Sage 1999)

Conclusions

Angiogenesis is a complicated multi step process involving a change in endothelial cell phenotype from quiescence to migration, involving the processes of tubulogenesis and endothelial cell proliferation. The aim of this study was to compare endothelial responses to a variety of known endothelial cell active factors in a stringent minimal medium system. This was primarily to answer the question about the relative effects of various pleiotropic factors and more or less specific endothelial factors with a view to promotion of endothelial cell migration, the key steps for graft take.

Of those assessed $TGF\beta_1$ and VEGF had a greater influence on endothelial cell migration than the fibroblast growth factors or interleukins. This work identifies $TGF\beta$ and VEGF as the best of the growth factors tested for stimulating endothelial cells from a quiescent state to a migrating invasive phenotype. However VEGF is known to be specific for endothelial cells whereas TGFß acts pleiotrophically.

The differences in response to different factors were less than two fold. The relatively poor response of endothelial cells to known angiogenic factors may reflect an alternating effect of the collagen matrix on migratory potential. Migration may require an optimal production of MMPs and excess of a factor may cause over production.

The results show that no single factor appears to stimulate a clinically dramatic migration effect. This finding in a single cell type *in-vivo* system corroborates what has been shown clinically with *in-vivo* wound healing, that no single factor appears to offer a "magic bullet" effect.

Chapter 5

The investigation of the mechanisms underlying endothelial cell migration with fibrin

5.1 Introduction

In chapter three, fibrin was identified as the extracellular matrix with the greatest ability to stimulate endothelial cell migration. This response was found subsequently to be greater than that elicited by any growth factor tested.

5.1.1 Fibrin structure and physiology

Fibrinogen is the target protein of the coagulation cascade, and following vascular injury, fibrin comprises the major protein component of the haemostatic plug. Fibrinogen is a 340 kDa soluble plasma protein consisting of three pairs of disulphide bonded α, β and γ chains.
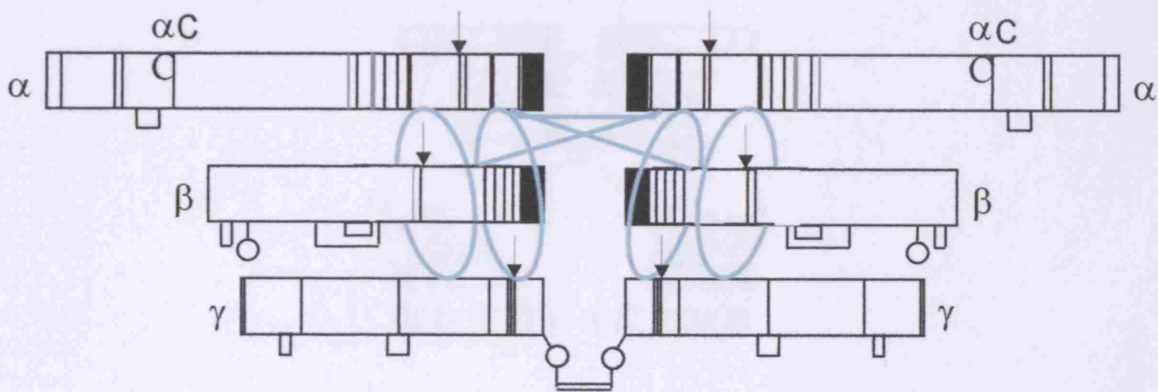
Figure 5.1 The structure of fibrinogen a 340 kDa soluble plasma protein showing cystein disulphide bridges (blue circles) and the cleavage sites giving rise to the major structural fragments D and E (black arrows)

The molecule consists of a central globular E domain connected by coiled regions to two identical globular D domains. In addition two other structures comprising approximately the carboxyl-terminal two thirds of the α chains, designated αC regions have been described. The E and D regions in each half-molecule are delineated by a pair of disulphide bridges, which link chains α to β, β to γ, and γ to α. Numerous sites at which plasmin (lines within the chains) can hydrolyse the molecule have been identified (vertical arrows above the chains). Thrombin converts fibrinogen to fibrin by catalysing the specific proteolytic cleavage of both fibrinopeptide A and fibrinopeptide B. These cleavages unmask two polymerisation sites in the E domain, forming soluble fibrin, to which one D region from each of two fibrinogen molecules can bind. The polymerisation of soluble fibrin to double stranded protofibrils is calcium dependent. The fibrin

monomers within each strand arrange end to end, and align in a half staggered overlap with the parallel chain.
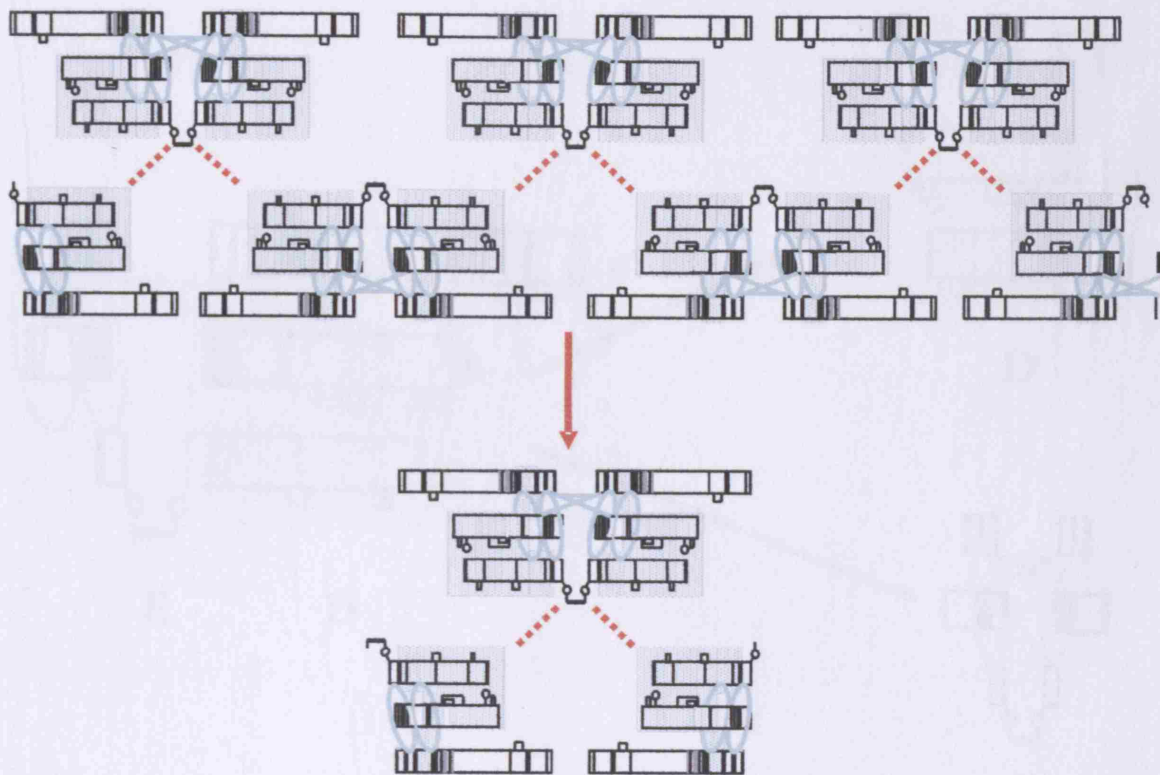
Figure 5.2 The polymerisation of fibrinogen to form a double stranded protofibril which is depolymerised by Plasmin cleavage

The protofibrils may further associate laterally to produce fibres which themselves may associate to form fibre bundles. Collectively the protofibril fibres and fibre bundles comprise the fibrin clot. The γ chains of adjacent D regions within a strand of a protofibril are covalently cross-linked by glutamine-glutamine peptide bonds catalysed by factor XIIIa. Thus, in cross-linked fibrin, as in a physical clot, the individual strands consist of polymers of covalently linked fibrin molecules. Factor XIIIa more slowly catalyses isopeptide bond formation in the αC region between multiple α chains within and presumably between protofibrils.

The dissolution of clotted fibrin results in specific hydrolysis catalysed by plasmin. The cleavage of fibrinogen and fibrin by plasmin has been extensively studied (Walker and Nesheim 1999). Fragmentation of fibrinogen occurs upon cleavages within the α chain to release the αC fragment, thereby producing fragment X (250 kDa). Further cleavage of fragment X in the α β and γ chains between the two disulphide bridges in one half of the

molecule produces fragment Y (160kDa) and fragment D (100kDa). Further cleavage of fragment Y produces a second fragment D and fragment E (60kDa, figure 5.3).
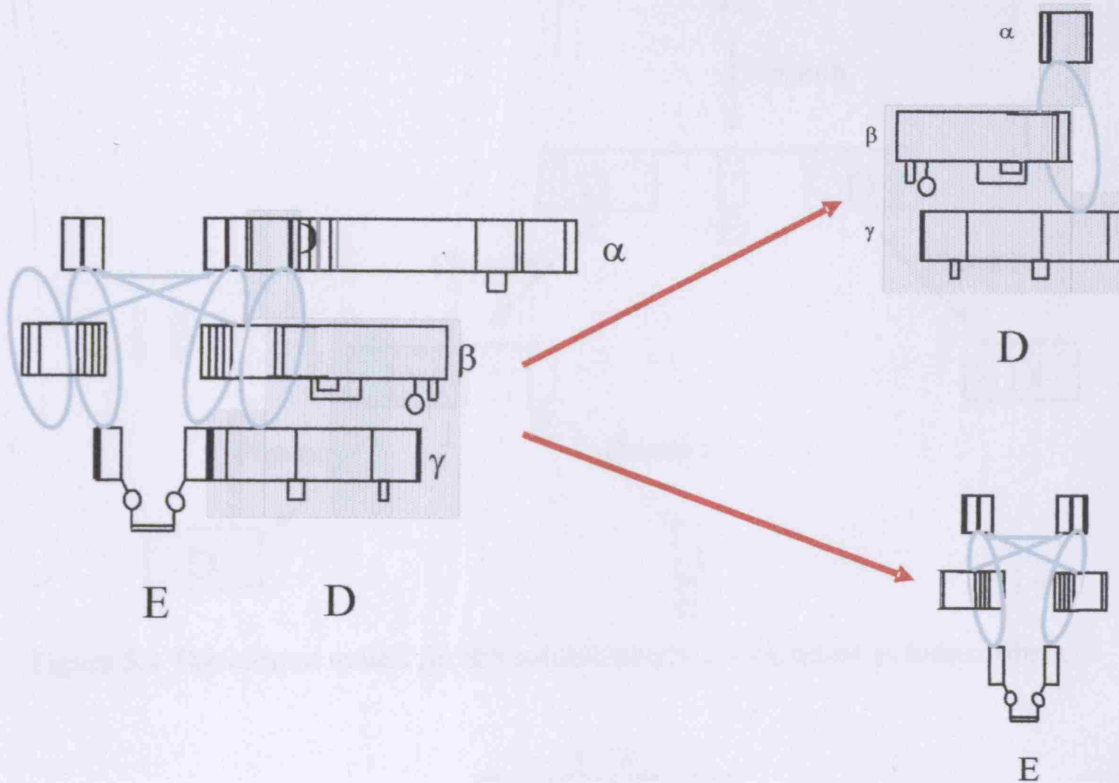
Figure 5.3 Cleavage of fragment Y generates both fibrin degradation products D and E
The terminal fragments E and D approximately comprise the respective E and D domains of the parent fibrinogen molecule.

The release of soluble material requires cleavage of the α, β and γ chains between the two disulphide rings in the region connecting the E and D regions of the fibrin monomers that are across from one another in the two strands of the protofibril. These are the same bonds that are cleaved in fibrinogen. Two sets of such cleavages must occur to obtain soluble products. Numerous soluble products can be produced, the nature of which depends on the relative locations of the sets of cleavages. Cleavages that yield a DD/E give the limit of digestion, larger products can also be produced DY, DXD, etc (figure 5.5).
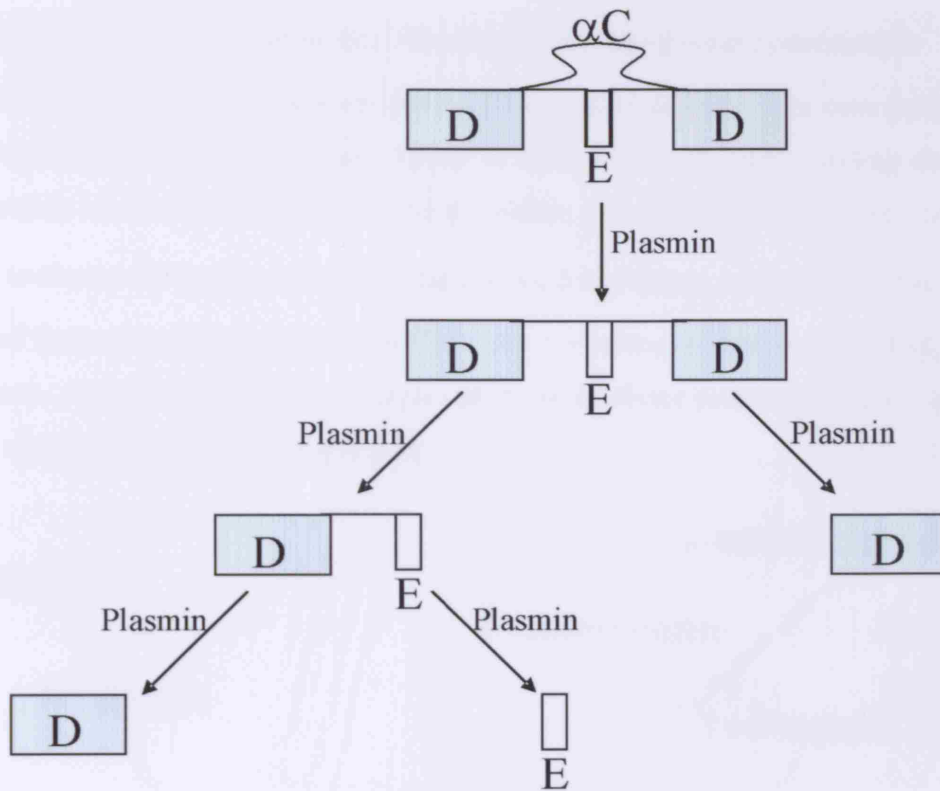
Figure 5.4 The current model for the solubilisation of γ chain cross linked fibrin.
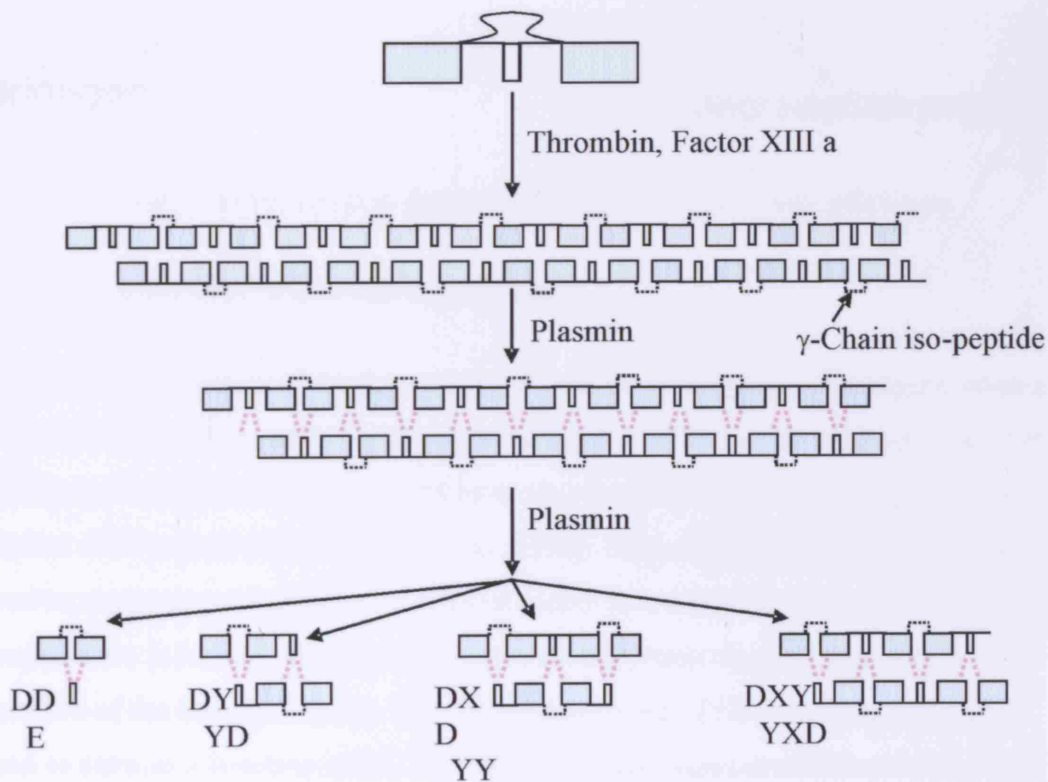
Figure 5.5 Summary of fibrin degradation into the sub units X, Y, D and E

Fibrin formation and degradation can be inhibited by anti-thrombotic agents (figure 5.6) acting at different points in the pathway of coagulation and fibrinolysis (Hirudin,

ε—Aminocaproic acid and Aprotinin). Hirudin is a 65 amino-acid cysteine-rich polypeptide produced by the salivary gland of the medicinal leech, it is now produced as a recombinant molecule. Hirudin binds tightly to thrombin at accessory binding sites and forms a stable non-covalent complex with thrombin, thereby abolishing its pro-coagulant function to cleave fibrinogen. ε—Aminocaproic acid is a lysine analogue that blocks the binding of tissue plasminogen activator (TPA) to plasminogen, hence preventing fibrin degradation. Aprotinin is a broad spectrum protease inhibitor with high affinity for plasmin which inhibits fibrin degradation.
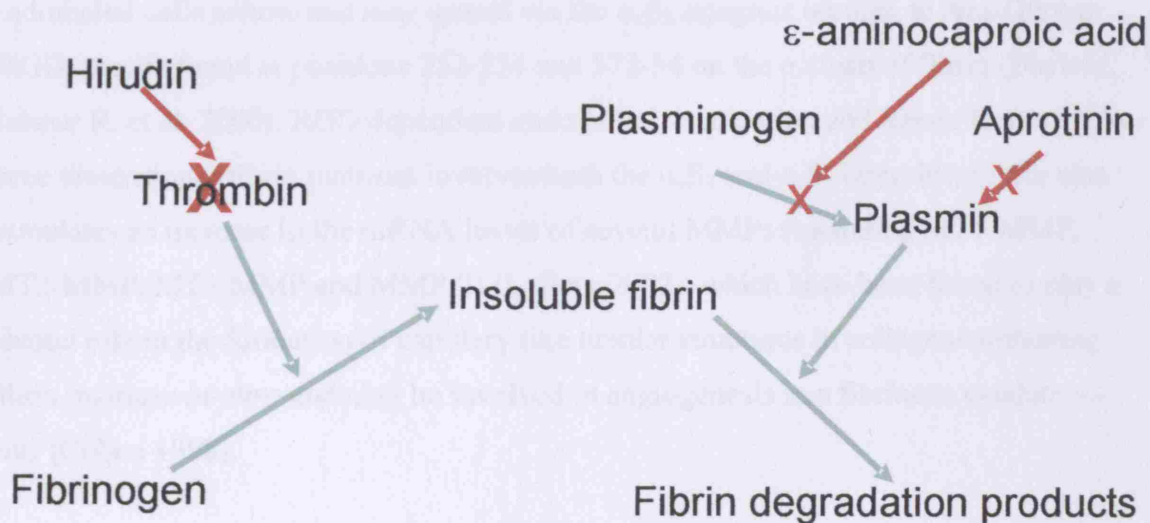
Figure 5.6 The enzymes involved in the formation of FDPs and their inhibitors

5.1.2 Fibrin/extracellular matrix and angiogenesis

The extracellular matrix (ECM) plays a crucial role in endothelial cell reorganisation and behaviour during angiogenesis (Folkman and Klagsbrun 1987; Ilan, Mahooti et al. 1998). Changes in the composition of the ECM have also been shown to be responsible for endothelial cell survival (Ilan, Mahooti et al. 1998). One of the native ECM components involved in angiogenesis is fibrin. Fibrin clots serve as a temporary matrix that provides a solid support for invading cells and disappears by enzymatic degradation during regeneration of the damaged tissue. The fibrillar structure of fibrin matrices has been analysed *in-vitro* as a function of H^+, Ca^{2+} and thrombin concentrations as well as the effect of hyaluronic acid on the polymer structure (Hayen, Goebeler et al. 1999). It has been shown that all these substances influence the fibril structure of fibrin, which may have considerable impact on angiogenesis during wound healing and graft revascularization (Collen 1998).

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Furthermore fibrin matrices have been utilised to investigate the angiogenic behaviour of different types of endothelial cells *in-vitro* in the presence of growth factors such as b-FGF, VEGF, PDGF, PD ECGF, HGF and IL-8 (Kumar 1998). The angiogenic potential of endothelial cells depends not only on stimulatory growth factors interacting with their appropriate cell surface receptors but on the proper interaction between cells and their ECM. These very specific interactions with the ECM are mediated by integrin receptors expressed on the cell surface

There are several potential mechanisms by which fibrin matrices stimulate angiogenesis. Endothelial cells adhere and may spread via the $\alpha_v\beta_3$ integrins binding to Arg-GlyAsp (RGD) motifs found at positions 252-254 and 572-54 on the α chain of fibrin (Bayless, Salazar R. et al. 2000). RGD dependent endothelial vaculoation and lumen formation in three dimensional fibrin matrices involves both the $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins. Fibrin also stimulates an increase in the mRNA levels of several MMPs (including MT1-MMP, MT2-MMP, MT3-MMP and MMP-2) (Lafleur 2002), which have been found to play a pivotal role in the formation of capillary like tubular structures in collagen-containing fibrin matrices *in-vitro* and may be involved in angiogenesis in a fibrinous exudate *in-vivo* (Collen 1998).

Collagen has conventionally been used as the basis for a Synthetic Dermal Equivalent and has shown its potential as a durable construct, its limitations in encouraging take and cellular ingress is becoming more evident. Results from chapter three show that fibrin provides by far the greatest impetus for endothelial cell invasion through an extracellular lattice. This chapter found that fibrin on its own promoted a 6.58 fold increase in endothelial cell migration when compared to collagen. The isolation of the active pro-angiogenic unit of fibrin or the angiogenic factor used in its manufacture may provide a further solution that could be simply added to a collagen matrix thus stimulating angiogenesis yet not disrupt the durability and pliability of a collagen construct. Results from chapter three do not explain whether the angiogenic response from fibrin was from coagulated fibrin, fibrinogen, a degradation product of these or possibly thrombin.

This chapter therefore aims to investigate;

1. The ability of each component of fibrin synthesis to stimulate endothelial cell migration with comparisons to fibrin and collagen.

2. The potential of the two major fractions of fibrin (D and E) in promoting endothelial cell migration.

3. The potential role of VEGF as a mediator

5.2 Materials and methods

In all the following experiments collagen was prepared at 0.75mg/ml and fibrin at 1.3 mg/ml (chapter 2.26). All experiments were replicated in at least three cell lines.

5.2.1 Experiment 1. The angiogenic analysis of the components of fibrin synthesis

Confluent cytoballs (chapter 2.24.) were plated in 400 μl gels of components as shown in table 5.1. Collagen was used at 0.75 mg/ml and fibrin at 1.3 mg/ml. The two gel matrices were prepared as described in chapter 2.2.7.1. All gels, once set, were bathed in a minimal serum media (MM).

Pilot studies were performed to assess how much plasmin was needed to macroscopically depolymerise the 400 μl of fibrin/collagen gel, and how much Aprotonin or EACA added subsequently was needed to inhibit this depolymerisation. Plasmin (0.1 units/ml), ε-Aminocaproic acid (EACA, 1mg/ml) and Aprotonin 100 units/ml) were added to the wells prior to gel addition. The media was exchanged after 48 hrs. The experiment was stopped and fixed at 96 hrs.

5.2.2 Experiment 2. Analysis of the angiogenic effects of thrombin

Confluent cytoballs were added to gels made from components shown in table 5.1. All gels were made in the manner described previously. Collagen, fibrin and collagen/fibrin gels served as controls. A pilot study showed that 1 unit of Hirudin was sufficient to inhibit the polymerisation of fibrinogen in the presence of added thrombin. Hirudin (1 unit) was placed into one well prior to the addition of the thrombin. Hirudin was also placed into a further well following full polymerisation of the fibrinogen with thrombin. MM was used as the nutritive medium in equal quantities to gel volume.

5.2.3 Experiment 3. The Effect of VEGF on migration in Fibrin/Collagen gels

Collagen and fibrin gels were prepared as per table 5.1. 400 μl of MM, 5% serum and 5ng/ml VEGF were added to separate collagen gels to serve as controls. To the Fibrin/Collagen gels; MM, 5% serum, 0.5ng/ml VEGF, 5ng/ml VEGF and 50ng/ml VEGF were added. Each medium was added in equal quantities to the set gel.

5.2.4 Experiment 4. The effect of VEGF receptor blockade on endothelial cell migration in fibrin gels

Collagen, with fibrin gels, were prepared and once added to the cytoballs they were plated and allowed to gel in 24 well plates. Minimal media with or without VEGF KDR receptor inhibitor ($1\mu M$, $3\mu M$, $10\mu M$, Calbiochem) was subsequently added to the plated gels as per table 5.1. All media was exchanged at 48 hours. The experiment was fixed at 4 days.

5.2.5 Experiment 5. The effect of VEGF neutralising antibody on endothelial cell migration in fibrin collagen gels.

Three collagen and two fibrin/collagen gels were prepared, added to cytoballs and plated, with or without thrombin, as appropriate. To the set collagen gels MM was added in equal quantities. To one of these gels VEGF 50 ng/ml was added, to another VEGF neutralising antibody was added (10 $\mu g/ml$) the other was left and served as a control. To the fibrin/collagen gels MM in equal volumes was added, one of which had VEGF inhibitor, the other left, acting as a control. All media with or without further components was exchanged at the 48 hours.

5.2.6 Experiment 6. The influence of fibrin degradation products D and E on endothelial cell migration.

Refer to chapter 2 for further details of the processes involved in this experiment.
100 mg Fibrinogen was prepared in 10 mls of Ca^{2+}, Mg^{2+} PBS. To this was added 0.8 units of thrombin to form a fibrin gel which was incubated for 60° at 37°C. Plasmin (4 units) was added after agitating and shaking the fibrin gel. After an hour of incubation the plasmin was subsequently inhibited using Aprotonin (4000 Units). The subsequent Fibrin Degradation Products (FPD) were stored at 4°C.

Following equilibration in running buffer and calibration of the Sephacryl S-100 high resolution 60 cm gel filtration column (chapter 2.3), FDPs (sterile filtered) were fractionated. Samples were taken according to corresponding trace peaks. These samples were refrigerated and pooled. The pooled samples were concentrated to 1 ml using ultrafiltration.

Table 5.1 The extracellular matrices used in each of the 6 experiments

Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
Collagen	Collagen	Collagen fibrin	Collagen	Collagen	Collagen
Collagen + Fibrinogen	Collagen + Fibrinogen	Collagen Fibrin VEGFR1 blocker 1mM	Collagen + 5% Serum	Collagen + VEGF 50 ng/ml	Collagen + fibrin subunit D 20 µg/ml
Collagen + Fibrinogen + Thrombin	Fibrinogen + thrombin	Collagen Fibrin VEGFR1 blocker 3mM	Collagen + VEGF 5ng/ml	Collagen + VEGF blocker	Collagen + fibrin subunit D 60 µg/ml
Collagen + Thrombin	Collagen Fibrinogen Thrombin and Hirudin (once gelled)	Collagen Fibrin VEGFR1 blocker 10mM	Collagen Fibrin + MM	Collagen + Fibrin	Collagen+ fibrin subunit D 200 µg/ml
Collagen Fibrin and Epsilon AminoCaproic acid	Collagen + Fibrinogen + Hirudin then Thrombin		Collagen Fibrin + 5% Serum	Collagen + Fibrin and VEGF blocker	Collagen+ fibrin subunit E 20 µg/ml
Collagen, Fibrin and Aprotinin			Collagen Fibrin + VEGF 0.5 ng/ml		Collagen+ fibrin subunit E 60 µg/ml
Collagen Fibrin and Plasmin			Collagen Fibrin+ VEGF 5 ng/ml		Collagen+ fibrin subunit E 200 µg/ml
			Collagen Fibrin+ VEGF 50 ng/ml		

Samples were analysed using SDS page stained with coomasie blue. The two major peaks were fractioned again by gel exclusion chromatography, the samples collected and run on SDS page gels under non-reducing condition in urea denaturing sample buffer. The fractions corresponding to molecular weights of 100-160 KDa (fraction D) and 100 – 37 KDa (fraction E) were stored (5°C). Fraction concentration was assessed by a Bio-Rad protein assay (chapter 2.5).

Each Fraction (D and E) was diluted to 40, 120 and 400µg/ml. These were added, each in equal quantities, to 200 µl of double strength collagen (1.5 mg/ml). Thus the end concentrations of the FDPs were halved to 20, 60 and 200µg/ml, and the collagen to 0.75 mg/ml. Cytoballs were added to these matrices as per previous experiments and the gels, after plating in 24 well plates, incubated for 1 hour to allow setting of the diluted 0.75mg/ml collagen. Once gelled, the *in-vitro* cytoball assay was incubated for 96 hours

with 400µl of MM per 400µl of FDP containing collagen. All media was exchanged after 48 hours and the fixed cytoballs assessed for endothelial cell migration.

5.3 Results

5.3.1 (Experiment 1) The angiogenic analysis of the components of fibrin synthesis

Components of fibrin manufacture added to collagen gels all stimulated endothelial cell migration more than the collagen control (p<0.05 Tukey, table 5.2) but thrombin-coagulated fibrinogen gave greatest effect. Migration was reduced further than in collagen only when plasmin was added to a collagen/fibrin gel (p>0.05 Tukey, figure 5.7). The collagen/fibrin matrix gave significantly more migration than any other matrix examined (p<0.05 Tukey). Collagen/fibrin matrices with EACA or aprotonin gave greater migration than collagen/fibrin matrices and plasmin (p < 0.05). The addition of thrombin to collagen stimulated an increase in migration (p < 0.05). There was no difference in migration provided by collagen and thrombin and that by collagen and fibrinogen.
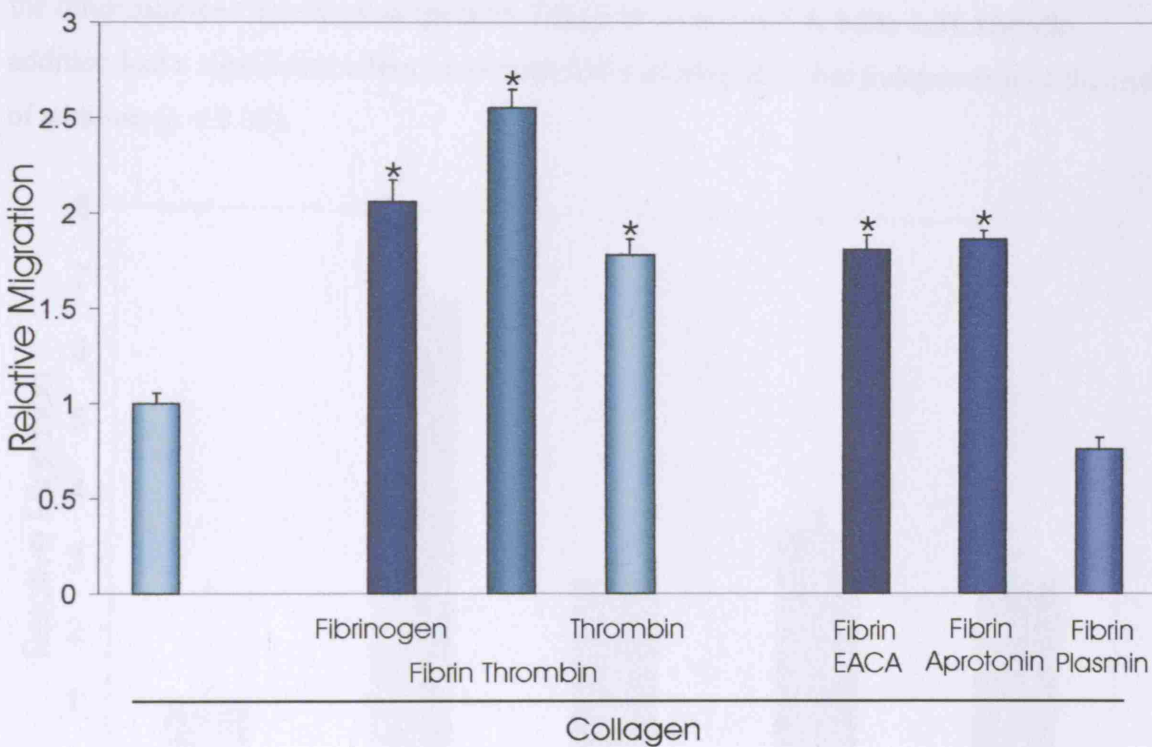
Figure 5.7 The influence of the components of fibrin manufacture on endothelial cell migration (mn +/- SEM, * p<0.05, Tukey n=3)

Table 5.2 Endothelial cell migration was greatest in collagen/fibrin gels with less migration in those gels containing products of fibrin degradation.

Test matrix	Relative migration to collagen
Collagen and fibrinogen	2.060
Collagen and fibrin	2.550
Collagen and thrombin	1.781
Collagen and Fibrin and EACA	1.800
Collagen and Fibrin and Aprotonin	1.860
Collagen and Fibrin and Plasmin	0.763

5.2.2 (Experiment 2) The potential of Thrombin to stimulate endothelial cell migration.

Fibrin stimulated a 6.73 fold increase in migration over collagen, this being greater than the other matrices investigated ($p<0.05$ Tukey, n=3, figure 5.8, table 5.3). Hirudin addition had a significant effect on endothelial cell migration but independent of the time of addition ($p < 0.05$).
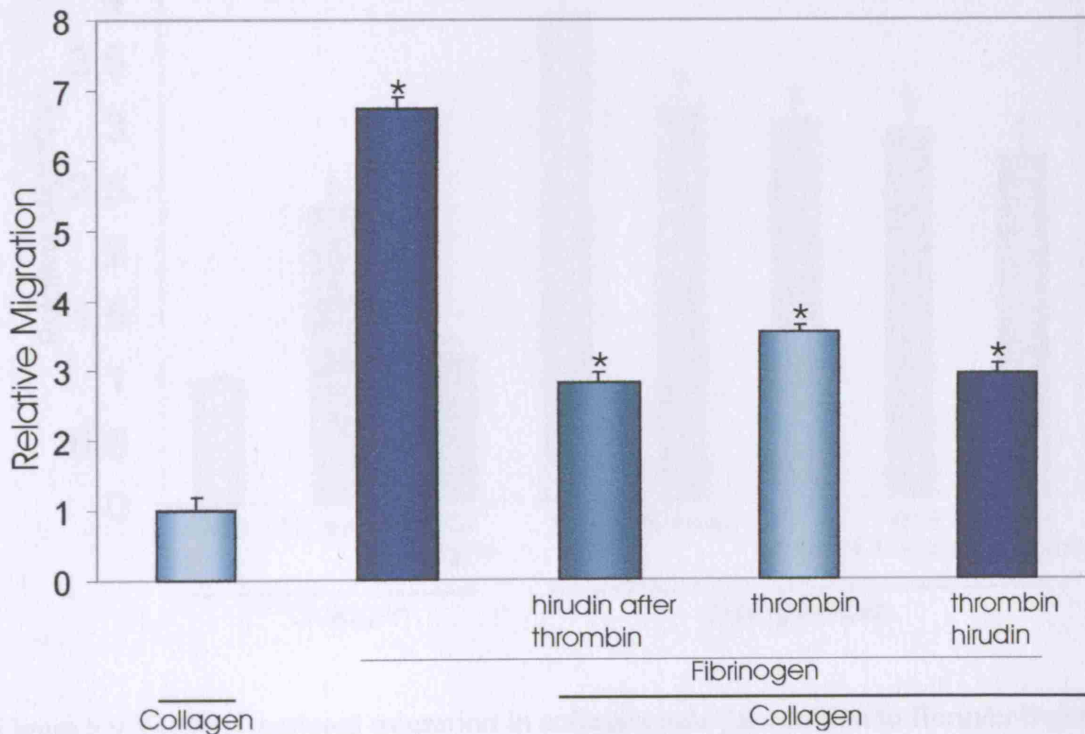
Figure 5.8 Hirudin addition decreased migration in collagen fibrin gels from 3.5 times that of collagen to 2.8 – 2.9 (mn +/- SEM, * $p<0.05$, Tukey n=3).

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Table 5.3 The greatest migration was seen with fibrin alone which was attenuated by collagen.

Extra Cellular Matrix	Relative migration to collagen
Fibrin	6.73
Collagen and fibrin	3.53
Collagen, fibrinogen and thrombin and Hirudin (prior to thrombin)	2.94
Collagen, Fibrin then Hirudin	2.83

5.2.3 (Experiment 3) Effect of VEGF on invasion of collagen/fibrin matrix

While the presence of growth factors (5% serum, VEGF) stimulated migration in collagen, they attenuated the migration in fibrin/collagen matrices (p>0.05, Tukey, n=3, figure 5.9)
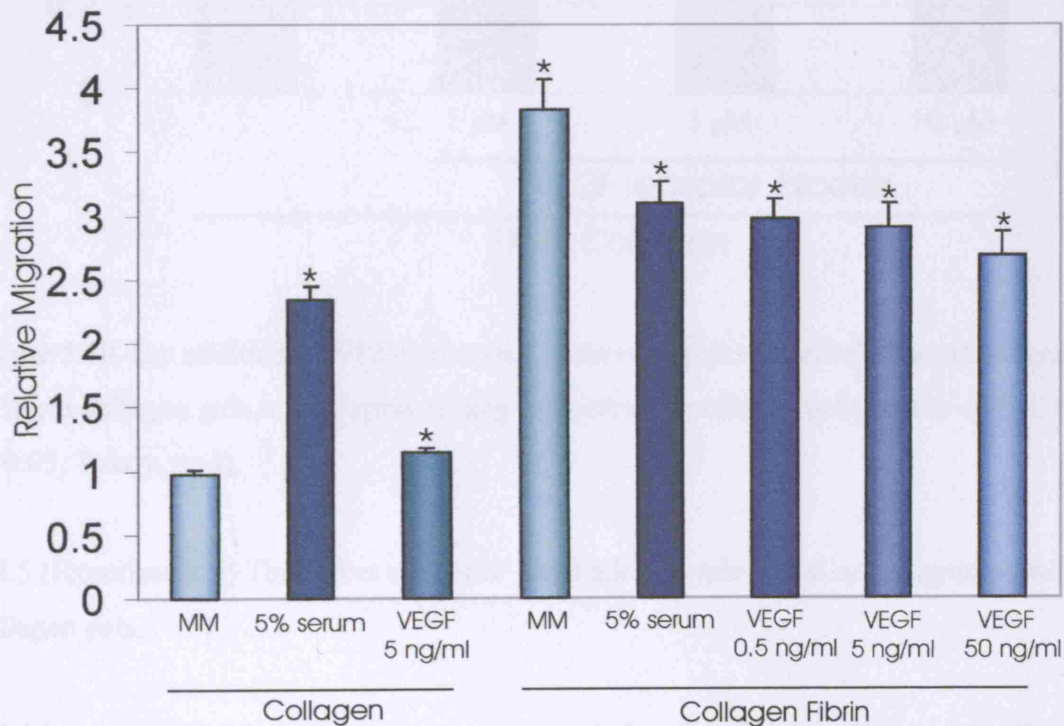
Figure 5.9 VEGF stimulated migration in collagen gels yet addition to fibrin/collagen matrices inhibited migration (mn +/- SEM, * p<0.05, Tukey, n=3)

5.2.4 (Experiment 4) The effect of VEGF receptor blockade on endothelial cell migration in fibrin gels

VEGF receptor blocker induced a concentration dependent decrease in endothelial cell migration in fibrin/collagen gels over the range tested. This was proportional to concentration. (p< 0.05 Tukey, n=3, figure 5.10). Receptor inhibition at a concentration of 10µM decreased the migration to 0.258 that of a fibrin/collagen control.
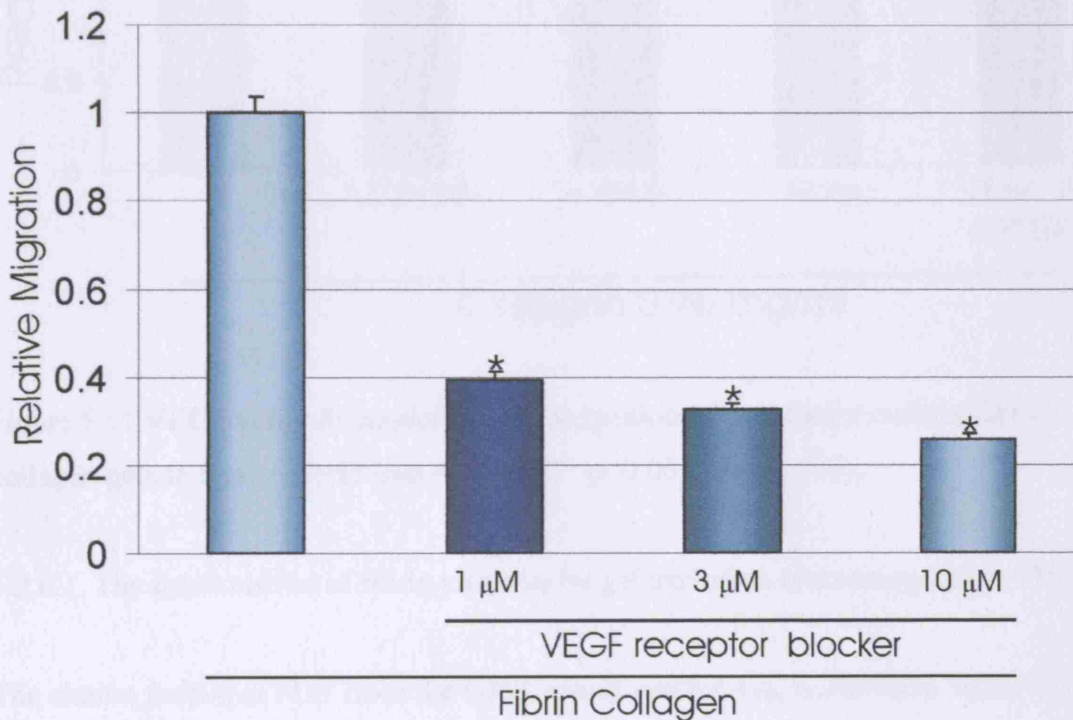
Figure 5.10 The addition of VEGF receptor blocker decreased endothelial cell migration in fibrin collagen gels to that approaching migration in collagen gels (mn +/- SEM, * p<0.05, Tukey, n=3).

5.2.5 (Experiment 5) The effect of VEGF blockade on endothelial cell migration in fibrin collagen gels.

Addition of VEGF 50ng/ml promoted an increase in migration in collagen gels. This effect was not seen with the concomitant addition of VEGF inhibitor, which gave an identical migration to collagen alone. Addition of VEGF inhibitor 1 to collagen/fibrin gels decreased relative migration from 2.54 to 1.07. This addition gave an identical migration value to that of collagen alone (p<0.05 Tukey, n=3, figure 5.11).

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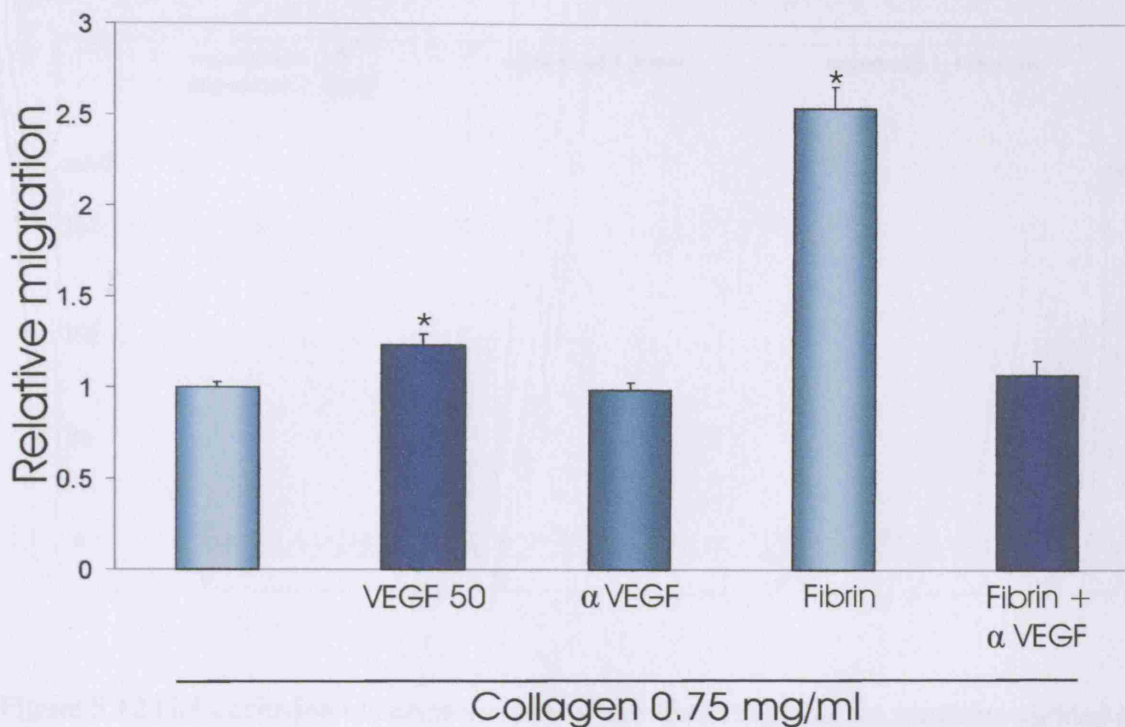
Figure 5.11 VEGF antibody moderated the migration of endothelial cells in fibrin collagen gels to that of fibrin (mn +/- SEM, * p<0.05, Tukey, n=3).

5.2.6.1. The fractionation of fibrin subunits by gel exclusion chromatography (GEC).

The elution profile of FDP from the GEC, monitored by A_{280} is shown in figure 5.12. The average time for complete elution was 110 minutes (132ml). Peaks were present from 44 to 120 mls. A single major peak roughly corresponded to the amylase calibration buffer (174 kDa). This peak was preceded by a single small peak and proceeded by numerous smaller peaks (figure 5.12).
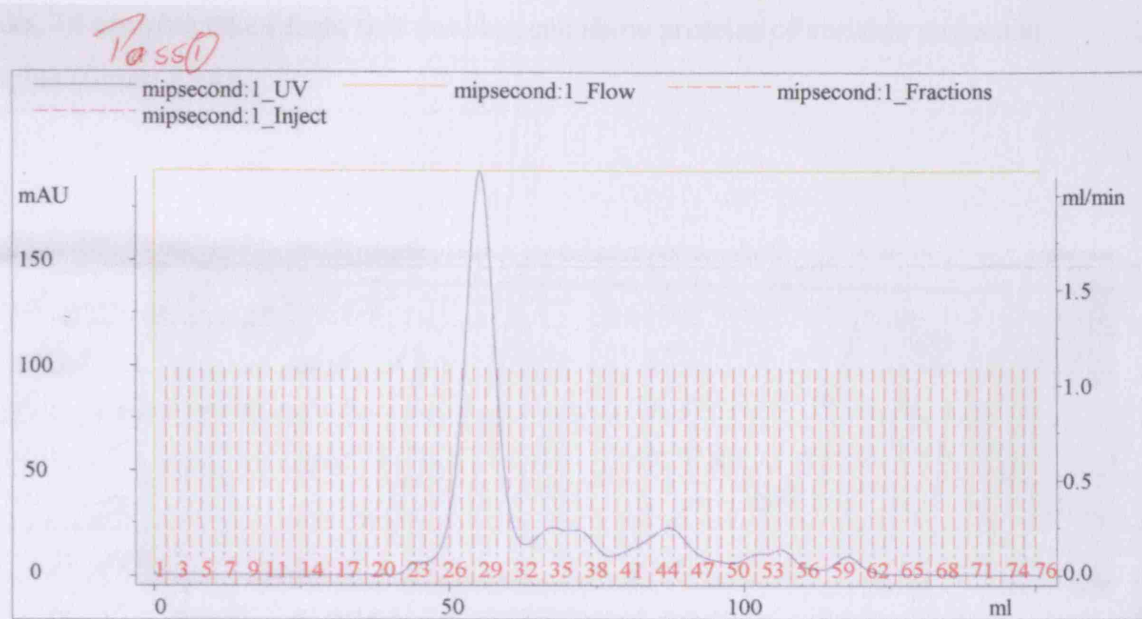
Figure 5.12 Gel exclusion chromatography of the fibrin degradation products yielded a trace with approximately eight peaks.

The collected samples corresponding to these peaks gave proteins of variable molecular weight on SDS page gels (figure 5.13).

Figure 5.13 the SDS page gel of the products of the Gel Exclusion Chromatography, numbered as per the GEC trace

The second peak gave proteins of variable molecular weights including those of 60 – 160 kDa (fibrin D and E).

Proteins pooled from subsequent GEC runs were collected corresponding to the 2nd peak (fractions 24 - 32). Further GEC of these pooled proteins gave a trace of 2 overlapping

peaks, 14 samples taken from this double peak show proteins of variable molecular weights (figure 5.14).
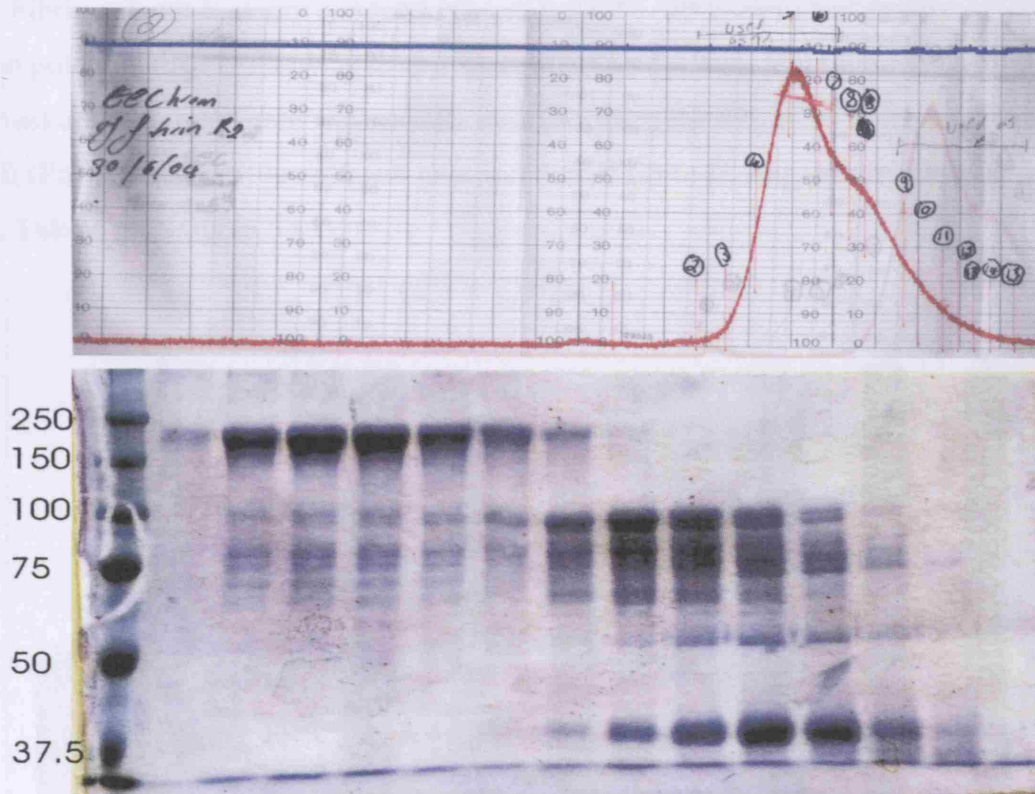
Figure 5.14 Gel exclusion Chromatography of the contents excluded from the second peak of the trace shown in figure 5.12 yielded a trace of two overlapping peaks (upper), SDS page gel of the14 samples generated form the GEC (lower)

This second GEC peak managed to further separate the proteins with the first six samples having predominantly higher molecular weight proteins (>150 kDa) and little of the lower molecular weight proteins (100kDa). The seventh peak contained both higher and lower molecular weight proteins however the following 8-12 samples had only lower molecular weight proteins, with prominent bands at 100 and 37 kDa. These proteins were pooled and sterile filtered and subsequently used in the cytoball assay.

5.2.6.2 The influence of endothelial cell migration with the fibrin degradation products D and E.

Addition of FDPs, D (100-160 kDa) and E (<100kDa) stimulated migration in a biphasic manner. Fibrin subunit E at any concentration tested (20 – 200μg/ml) had greater migration potential than D likewise at any concentration. Greatest migration with either subunit was at 60 μg/ml. There was no difference between the migration given by fibrin subunit E (FnE) at 60μg/ml in collagen gels and that of fibrin (1.3mg/ml) in collagen gels. (p<0.05, Tukey, n=3, figure 5.15).
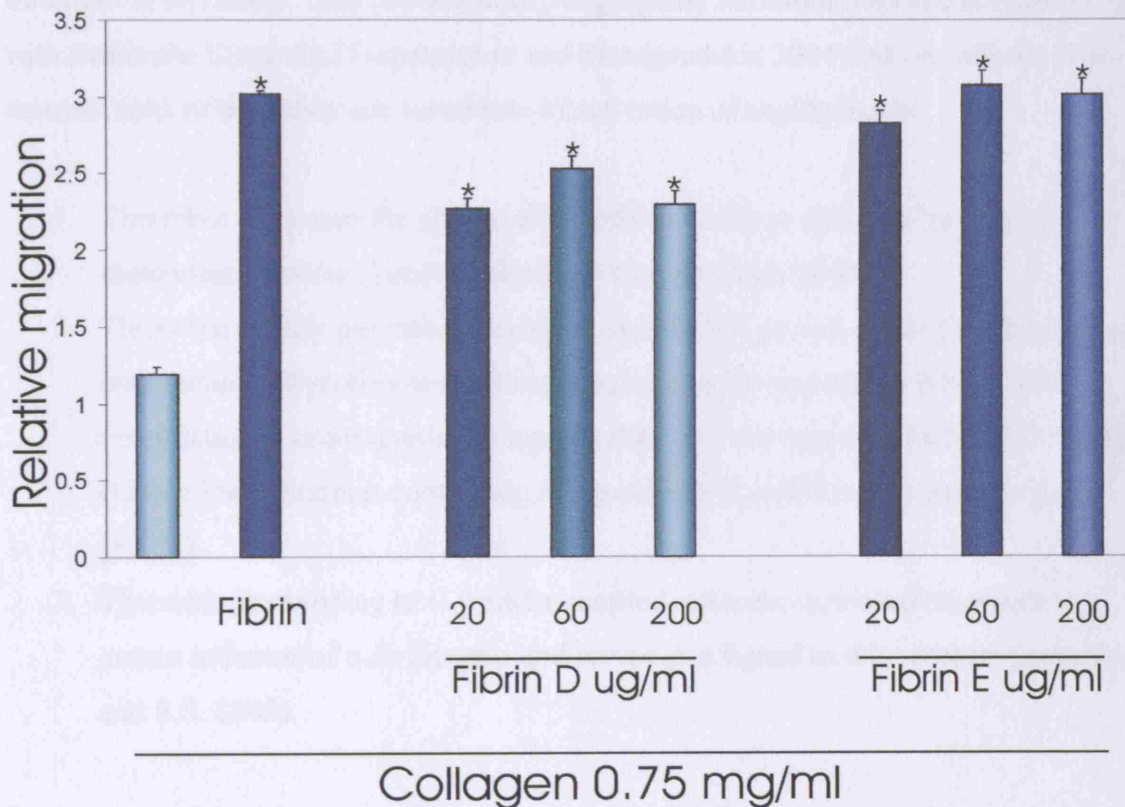
Figure 5.15 Both fibrin subunits D + E stimulated endothelial cells to migrate in a biphasic concentration dependent manner compared to collagen alone (mn +/- SEM, *p<0.05, Tukey, n=3).

5.3 Discussion

This chapter presents work to investigate the mechanism by which fibrin imparts its strong response on endothelial cell migration.

The addition of thrombin to collagen stimulated an increase in migration by a factor of 1.781 over collagen alone. The effect of thrombin could not account for the effect of fibrin. Hirudin blocks the proteloytic activity of thrombin, but had a small effect on thrombin's stimulatory activity. This is consistent with a non-proteolytic mechanism of thrombin in this assay. This pro-migratory/angiogenic thrombin response concords well with that in the literature (Tsopanoglou and Maragoudakis 2004). At the cellular level many actions of thrombin can contribute to activation of angiogenesis:

1. Thrombin decreases the ability of endothelial cells to attach to basement membrane proteins (Tsopanoglou and Maragoudakis 2004).
2. Thrombin greatly potentiates vascular endothelial growth factor (VEGF) induced endothelial cell proliferation (Tsopanoglou and Maragoudakis 2004). This potentiation is accompanied by up-regulation of the expression of VEGF receptors (kinase insert domain-containing receptor [KDR] and fms-like tyrosine kinase [Flt-1]).
3. Thrombin, by binding to G protein-coupled, protease-activated receptors, is a potent activator of $\alpha_v\beta_3$ integrin and serves as a ligand to this receptor (Stouffer and S.S. 2003).

Furthermore, thrombin increases the secretion of VEGF and enhances the expression and protein synthesis of matrix metalloprotease-9, MMP-2 (Maragoudakis, Tsopanoglou et al. 2002) and $\alpha_v\beta_3$ integrin(Tsopanoglou and Maragoudakis 2004). While no other study has directly compared thrombin, collagen and fibrin for their ability to stimulate endothelial cell invasion, many centres have noted thrombin's effect to stimulate migration *in-vivo* with many commenting on the need for PAR1 expression for such stimulation. Archiniegas noted that this response was related to the PAR1 receptor expression (Archiniegas 2004). Our *in-vitro* study measured cellular migration, this positive response agrees with that in the literature. Thrombin however may well have conflicting mechanisms of action on other processes in angiogenesis. Chan found that through PAR1 receptor activation thrombin inhibited endothelial cell tube formation (Chan 2003).

The addition of hirudin, blocking thrombin polymerisation of fibrinogen, dramatically decreased the migration of endothelial cells, in fibrin/collagen gels whether added prior to fibrinogen polymerisation or after fibrin formation. Hirudin binds to two distinct sites on thrombin, its active (catalytic) site and its fibrinogen-binding site (exosite 1) (Warkentin 2004). Clinically it has been used as a potent direct thrombin inhibitor and has provided excellent antithrombotic efficacy (often associated with a significant risk of hemorrhage) (Haas 2003). As an angiogenic agent it has been found to inhibit neo-angiogenesis in the chick chorioallantoic membrane over a 24-72-h period by approximately 2-3-fold, this by ligation of the thrombin protease-activated receptor, PAR-1 (Caunt 2003). In the cytoball assay hirudin addition after thrombin inhibited migration to the same extent as when added before. This intimates that thrombin may have some action independent to fibrin. The angiogenic response seen with fibrin/collagen gels in experiment 1 may, in part, be due to thrombin acting directly on PAR1 receptors to induce a proportion of the endothelial cell migration seen.

The addition of inhibitors of plasminogen activation (ε–Amino Caproic Acid (EACA) and aprotonin) caused an identical decrease in endothelial cell migration when added to collagen/fibrin gels. They increased responses relative to fibrinogen as well as to fibrin. If EACA had no effect but aprotinin blocked migration this would suggest exogenous plasmin. Because the effects were identical endogenous plasmin could well be involved. The decreased migration could be for the following reasons;

1. Both aprotonin and EACA impart an anti-angiogenic response
2. The normal response to fibrin requires gradual and local degradation of fibrin by cellular plasmin.

No study has investigated and compared the direct effects of EACA and aprotinin *in-vitro* on endothelial cell invasion/migration through ECM gels. Nevertheless EACA is known for its anti angiogenic properties (Ambrus 1992) and inhibiting matrix deposition (Kraling 1999). Aprotinin, a serine protease inhibitor, often through its inhibitory effects on plasmin, inhibits endothelial cell migration through ECM gels *in-vitro* (Strand 2000).

The addition of soluble angiogenic growth factors to collagen gels produced a clear but modest stimulation of migration, with VEGF promoting invasion to 1.4 times that of a

minimal media. The addition of VEGF to fibrin collagen gels attenuated the migration seen with identical gels with MM stimulation. This may be due to competitive blocking pathways or VEGF concentrations reaching inhibitory levels, as shown by the migration growth factor concentration profile of bFGF and IL8 in chapter 4. A similar study using micro-carriers of endothelial cells found contradictory results (Sun 2004). Using solitary fibrin gels of 1mg/ml supplemented with EGF and hydrocortisone, without collagen, VEGF was found to stimulate endothelial cell sprout formation through the gels. While this *in-vitro* assay differed slightly in extracellular matrix and did not specifically measure individual cellular migration, its results oppose those in this chapter.

This work provides strong evidence that the "fibrin" response is mediated by autocrine VEGF production. VEGFr, a selective KDR inhibitor with a reported K_i of 100nM, was used over a concentration range to provide between 10-100 times this value to limit non-specific activity. Endothelial cell migration was inhibited proportionally to the concentration of receptor blocker applied. 10 μM of receptor blocker decreased migration within a fibrin/collagen gel to that of a collagen gel, in effect blocking the migratory influence of fibrin. This agrees with likewise studies exploring the migration, proliferation and tubulogenesis of endothelial cells in fibrin gels which concluded that inhibition of VEGF receptors significantly decreased the endothelial cell response seen in fibrin gels (Nakatsu 2003). This is hard to reconcile with the attenuation of migration in collagen/fibrin seen with VEGF addition. In using a VEGF-blocking antibody a similar concentration dependent decrease in migration was seen in fibrin/collagen gels. This reduced the migratory effect of fibrin to levels statistically similar to that of collagen. Taken together these results suggest a plausible mechanism that fibrin stimulates autocrine VEGF release. This data concurs with *in-vivo* studies examining the degree of VEGF induced by fibrin based dermal substrates. Hojo found that fibrin based dermal replacements showed an excellent take rate *in-vivo* and found that the most likely cause for this improved take rate over conventional collagen based materials was a three fold induction in VEGF$_{121 and 165}$ from the surrounding donor tissue (Hojo, Inokuchi et al. 2003). An hypothesis for inhibitory effect of exogenous VEGF could be that high concentrations stimulate plasminogen and PA expression or MT-MMPs which result in excessive fibrinolysis. This could be examined in further experiments.

The Gel Exclusion Chromatography curve of FDPs show partial separation of D and E fragments. The reasons for initial low resolution may be;

1. The intrinsic resolution of the column is inadequate to separate molecules (figure 5.14)

2. The subunits interact in solution

3. D has a seven fold larger absorbance and is in excess of E by two fold.

Our results corroborate some early research performed on fibrin degradation products and their chromatographic elution, showing a lack of clear separation of the fibrin degradation products (Mihalyi and Towne 1976). It is clear, from SDS page gels of samples taken from the second GEC peak, which coincides with a mass of approximately 170-190 KDa, that initial samples from the peak contain fragment D and a trace of Y. Following fractions contain mostly D with the relative proportion of fragment E increasing steadily with a rapid decrease in D. This relationship is identical to that shown in the trypsinisation of fibrinogen using identical methods (Mihalyi and Towne 1976). The fact that both of these fragments were separated by further GEC of the second peak suggests that there is no appreciable interaction between both fibrin fragments D and E. The inability of the first GEC to separate these fragments has been shown to be due to fragment D having approximately seven times greater A280 than that of E (due to more tyr and phe residues) combined with the intrinsic limitation of resolution afforded by the sephacryl medium.

The capacity of fibrin fractions E (FnE) and D (FnD) to stimulate endothelial cell migration has been shown in this *in-vitro* assay. It has proved that fraction E at identical concentrations stimulates greater endothelial cell migration than fraction D. The response to E at the optimum concentration tested is equal to that of fibrin. Proangiogenic effects of FnE are well documented, it stimulates neovascularisation in chick chorioallantoic membrane assays of angiogenesis (Thompson 1992) and enhances the outgrowth of smooth muscle cells from rabbit aortic medical explants in culture (Naito 2000). Similar *in-vitro* studies using 10- 100nM FnE stimulated an increase in dermal endothelial cell proliferation over and above that of VEGF. Migration across collagen IV gels with FnE was found to be 32.3% greater than bFGF and 51.4% greater than VEGF ($1\mu M$ FnE was inhibitory). This promigratory effect agrees with that found in our study, however using the cytoball assay, migration with FnE was approximately twice that of VEGF. FnE also stimulates tubule formation in matrigel *in-vitro*. However in this instance such stimulation was significantly increased by VEGF or bFGF addition (Bootle-Wilbraham 2001).

This work is important because it shows that the "fibrin effect" in collagen can be replicated by a soluble fragment. This proves that fibrin degradation could be necessary for the "fibrin effect" at least in large part. It is consistent with the EACA/aprotinin result. This is possible because the assay allows a direct comparison between effects of a solid matrix and a soluble material. Hence this extends the scope of previous work on fibrin E.

Conclusions

This chapter has given considerable evidence as to the mechanism of the pro-angiogenic response to fibrin. These results suggest that fibrin degradation may be relevant to the use of fibrin as a scaffold and that FDPs may themselves be a useful material for incorporating into scaffolds. It also has considerable implications in the field of anti-angiogenesis and oncology. It has explored the angiogenic properties of fibrin as a pro-migratory stimulus to endothelial cells. It indicates that fibrin degradation is an important process in the fibrin response and also that the response is mediated by an autocrine stimulation of VEGF.

These results suggest the use of fibrin degradation products as potential pro-angiogenic components in a second generation dermal skin equivalent. The use of smaller fragments that impart a strong angiogenic stimulus yet have little effect on the structure of the matrix may provide alternative strategies in bioengineering a pro-angiogenic matrix.

Chapter 6

The influence of ultrasound on endothelial cell invasion into matrices

6.1 Introduction

The experimental aims of work described in this chapter were to explore whether Ultrasound could stimulate angiogenesis *in-vitro* and investigate the optimum intensity of any effect for potential clinical use. This introduction gives a brief review of the mechanisms of action of ultrasound and its current significance as an adjunct to wound healing.

Ultrasound is inaudible high frequency vibration created by electrical energy deforming a piezo electric crystal with the resultant excitation generating mechanical energy. Energy leaves a generator probe and is conducted through tissue by propagation, vibration and collision, with a progressive loss of energy due to absorption, dispersion and attenuation.

Ultrasound can be regulated by beam intensity (W/cm^2), frequency and pulsatility. The amount of energy that reaches a specific target depends on the intensity, amplitude, beam focus and frequency as well as the absorbancy of the tissues through which it travels. Lower tissue absorbance (impedance) and thus higher penetration are seen in tissues of high water content. The greater the difference in impedance at a boundary, the greater the reflection that will occur and therefore the smaller the amount of energy transferred (table 6.1). The difference in impedance is greatest for a steel/air interface. This is overcome by the use of a coupling agent. While most US machines provide intensities from 0.1-3 W/cm^2 the optimum intensity for wound healing is unknown. Most studies use intensities between 0.1-1.0 W/cm^2 (Lundeberg 1990), nevertheless there has been no direct comparison on wound healing outcome between intensities.

Table 6.1 The acoustic properties of different materials with US transmission

Material	Velocity (m/s)	Density (kg/m^3)	Impedence ($kg/m^2/s$)
Air	340	0.625	213
Bone	2800	1800	5.1×10^6
Fat	1450	940	1.4×10^6
Muscle	1550	1100	1.7×10^6
Steel	5850	8000	47×10^6
Water	1500	1000	1.5×10^6

Therapeutic ultrasound has a frequency range of 0.75-3MHz. Low frequency waves (1MHz) have less penetration and thus are commonly used for superficial effects such as wound healing, conversely higher frequencies (3MHz) have greater penetration but are less focussed (Johnson 2003). Ultrasound can be continuous or pulsed, the latter allowing heat dissipation during treatment. Though no study has specifically investigated the ratio of on-time to off against wound healing trajectory there is a consensus that a regime of 2 milliseconds on, 8 milliseconds off is effective in the treatment of chronic wounds (Dyson and Franks 1976).

Therapeutic effects of US are generally divided into two main categories: thermal and non-thermal.

6.1.1 Thermal

Thermal effects of US occur when tissue temperature is elevated to 40-45°C for at least 5 minutes. They include increased blood flow, reduction in muscle spasm, increased extensibility of collagen fibres and a pro-inflammatory response (Dyson 1978). Excessive thermal effects associated with high intensities damage tissue (Dyson 1987). Among the more effectively heated tissues are periosteum, superficial cortical bone, collagenous tissues (ligament, tendon and fascia) and fibrotic muscle (Dyson 1968). Most authorities nevertheless attribute a greater importance to the non-thermal effects of US on wound healing.

6.1.2 Non Thermal

The non-thermal effects of US fall into 3 main categories: cavitation, acoustic streaming and micro-massage.

Cavitation is the formation of gas filled voids within the tissue and body fluids. There are two types of cavitation, stable and unstable. Stable cavitation is the formation and growth of gas bubbles from dissolved gas in the medium. They take approximately 1000 cycles to reach their maximum size (Wells 1977). The cavity acts to enhance acoustic streaming phenomena and as such would appear to be beneficial. Unstable cavitation (transient) is the formation of bubbles at the low-pressure part of the ultrasonic pressure wave. These

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bubbles collapse releasing large amounts of energy, a process that is detrimental to tissue. There is no evidence at present to suggest that this phenomenon occurs at therapeutic levels.

Acoustic streaming is described as a small scale eddying of fluids near a vibrating structure such as cell membranes and the surface of a stable cavitation gas bubble (Dyson 1978). It is due to the mechanical pressure changes within the US field.

Table 6.2 Randomised controlled trials investigating the effect of ultrasound on venous ulcers

Author	Patient number	Treatment regime	Results
(Eriksson, Lundeberg et al. 1991)	38	1MHz of US for 10 mins twice weekly for 8 weeks	32% US treated ulcers healed at 8/52, 21% with sham US control
(Lundeberg 1990)	44	1MHz of Us for 10 mins three times weekly for 4/52 then twice weekly for 4/52, then weekly unless healed	45% healed in 12 weeks with US, 36% healed with sham US control
(Roche and J. 1984)	26	3 MHz three times per week for 4/52 with 8/52 follow up.	35.3% decrease in ulcer size with US group, 6.96% with sham US group
(Dyson and Franks 1976)	25	3MHz for 10 mins three times a week for 4/52. wounds assessed for percentage of initial wound area present at 4/52	66.4% ulcer left with US group, 91.6% with the sham US controls (p < 0.05)
(Callam 1987)	108	1 MHz for 1 minute, once a week for 12/40	61% ulcers healed with US at 12 weeks, 41% healed with standard therapy

6.1.3 Ultrasound and wound Healing

Much research on US and living tissue has been extrapolated from *in-vitro* studies, to focus on wounds/ulcers. There is some evidence that US can influence several component processes of inflammation by stimulating the production of macrophage-derived fibroblast mitogenic factors (Young 1990), accelerate fibrinolysis (Francis 1992; Harpaz 1993) and increase fibroblast migration (Young 1990). US can also stimulate healing processes such as angiogenesis (Young 1990), matrix synthesis with more collagen synthesis and increased tissue tensile strength (Byl 1992); (Byl 1993)'. This research has provided the basis for US as an adjunct in wound healing.

Table 6.3 Randomised controlled trials investigating the effect of ultrasound on decubitus ulcers.

Author	Patient number	Treatment regime	Results
(McDiarmid 1985)		3 Mhz for 5 mins three times per week.	48% healed with US, 42% with sham therapy
(Ter Riet, Kessels et al. 1995)	88	3.28MHz five times per week for 12/40 or until healed	40% healed with US, 44% with control therapy
(Nussbaum, Biemann et al. 1994)	16	Combination of US and ultraviolet treatment or laser therapy (820nm laser diode) given alternately for five days every week, or standard wound care	All sores healed with US group, 66% healed with laser therapy, 83% healed with standard wound care.

6.1.4 Ultrasound therapy and clinical studies

There is considerable *in-vivo* and *in-vitro* data demonstrating that ultrasound stimulates healing processes yet the few adequately controlled clinical trials reporting an objective change in a measured parameter are conflicting. Studies have been confined to venous

and decubitus ulcers with outcomes measured as time to complete healing, proportion of ulcers healed and rate of change in ulcer area.

With venous ulcers, of the published randomised controlled trials to date (table 6.2), results suggest that ultrasound improves venous ulcer healing rate. The majority of the trial designs were of low power and hence limited statistical significance. Similarly, small patient numbers have compromised randomised studies comparing US to control therapy with decubitus ulcers. Two trials compare ultrasound therapy to a sham therapy and one compares US, ultraviolet light therapy and laser treatment to a sham treatment (table 6.3), their results are conflicting. Pooling the data from the first two trials due to their similarity allows the assessment of US in 128 patients. Such data shows that there is no evidence of a benefit on ultrasound on the healing of decubitus ulcers.

6.1.5 Effects of Ultrasound on cell proliferation, protein synthesis, cytokine production and angiogenesis

The molecular mechanisms by which ultrasound alters cell function or protein synthesis are still not known. The following processes have been implicated;

1. Mechanical forces may act on cytoskeletal attachments which can trigger integrin signalling, affecting cell metabolism and gene expression (Yang 1996).

2. Changes in the cell membrane permeability with enhanced secondary messenger activity. This is thought to occur by; cavitation, producing oscillatory movement of micro-bubbles, the compression of microtubules and acoustic streaming.

3. Electrical currents in bone may be potentiated by exposure to ultrasound energy. Investigators have reported increased potentials as a function of ultrasound intensity and frequency (Behari 1981; Duarte 1983).

4. Ultrasound generation of mechanical forces, as hypothesised with VAC therapy (Argenta and M.J. 1997), could activate "stretch receptor" type cation channels (Sachs 1991) with modification of intracellular signals, regulating gene expression.

5. Thermal effects will give an hyperaemia with a resolution of inflammation and increased cell metabolism (Webster 1980).

Ultrasound has been shown to stimulate, in *in-vitro* studies, the synthesis of angiogenic related cytokines; Il-8, bFGF, VEGF and Il-1 with a resultant increase in collagen

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production and angiogenesis (Reher 1999) (Doan 1999). Only one study has quantitatively investigated the effect of US on angiogenesis in wounds. Young observed the rate of new blood vessel formation in full thickness excised lesions on the flank of adult rats (Young 1990). Wounds exposed to ultrasound daily for 5 minutes (0.1 W/cm^2 at 0.75- 3.0 MHz) at five days had significantly more vessels that sham treated controls (p<0.05), with 30% more blood vessels with 0.75 MHZ treatment and 20% more with 3.0 MHz treatment. Significantly US was shown to accelerate vascularisation but not to cause a pathophysiological change in vasculature, with no difference observed between US treatment and controls at seven days.
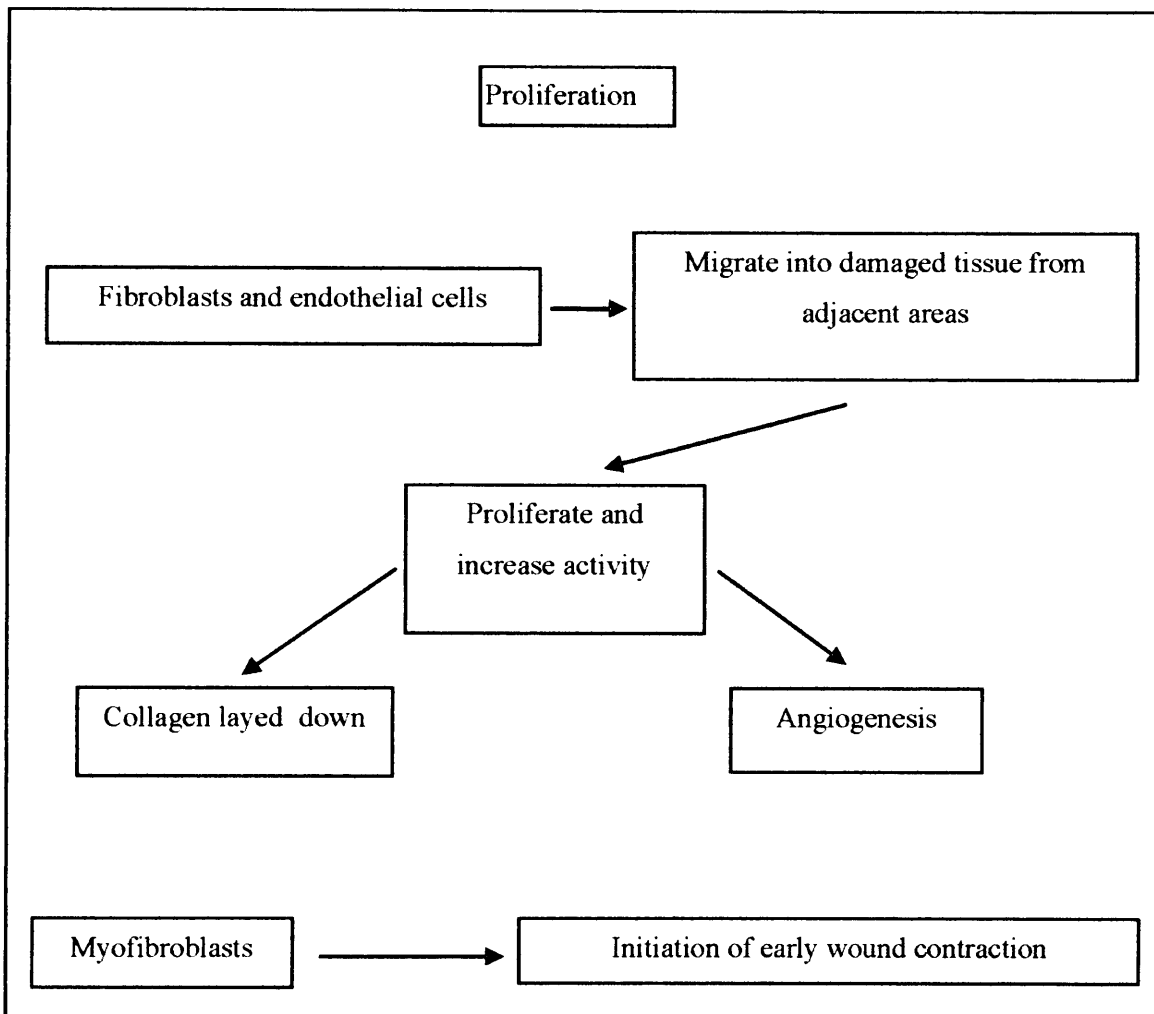
Figure 6.1 Effects of ultrasound on wound healing

The macroscopic and molecular mechanisms underlying such angiogenic responses are complex. However much research has focused on growth factor induction by varied cell types in response to US. Osteoblastic studies have shown that US induces VEGF –A transcription mediated by HIF -1α and Nitric Oxide (Wang 2004).

Further studies have shown that this US-induced VEGF stimulation occurs not only in osteoblasts but also in monocytes and fibroblasts (Reher 1999). IL-1β production is stimulated in these cells in response to US albeit to a lesser degree (Reher 1999). Whilst these studies may account for the enhanced angiogenesis described above no studies have investigated only direct responses of endothelial cells directly to US.

This chapter aims to investigate whether US can stimulate angiogenesis directly. The previously validated cytoball assay was used to quantify the effect of ultrasound on the stimulation of endothelial cells from a quiescent to an activated migrating phenotype, through an extracellular matrix from a fixed point.

6.2 Materials and methods

Endothelial cells from human placentae were cultured *in-vitro* using media containing 5% serum (chapter 2.2.3). Cells were co cultured with cytodex beads for 24 hours. Once cell growth was confluent around the bead circumference, the beads were evenly mixed with 0.75 mg/ml collagen and plated in 24 well plates. Approximately thirty beads were used in three wells for each plate and each cell line used two plates, one for ultrasound and one for control. Once gelled (30 minutes), equal volumes of minimal media were added to the matrix.

Pulsed ultrasound (2ms on, 8 msec off) of 3MHz at 0.2, 0.8 and 3W/cm^2 was applied in a circular fashion to the base of the plate using a coupling gel. The media and gel were incubated at 37 °C with 5% CO_2 for 96 hrs, all media was exchanged at 48 hrs and the gels fixed (4% Paraformaldehyde) at 96 hrs.

Cytodex beads were photographed and the average cell invasion distance from each bead calculated. These values were collated and thus a mean migratory distance for either US or control obtained per cell line. Relative migration compared to the control conditions allowed amalgamation of data from all three-cell lines to give a comparative value of migration provided by Ultrasound.

6.3 Results

Confluent cell cultures on cytodex beads showed sprouting, substratum detachment and three-dimensional invasion within collagen gels (illustrated at 96 hrs, figure 6.2A). This behaviour was consistent in all cell isolates studied. All cell lines showed a strong response to Ultrasound at 0.8w/cm^2, increasing endothelial cell invasion within collagen gels over control conditions (Dunn's, $p < 0.001$, figure 6.3, table 6.4).

Cell line	Invasion with US (microns)	Invasion in control gels (microns)
N 5	129.29	47.43
N 6	124.46	31.47
N 7/7	92.66	39.18

Table 6.4 Invasion of three endothelial cell lines in collagen gels with or without Ultrasound stimulation (0.8W/cm^2)
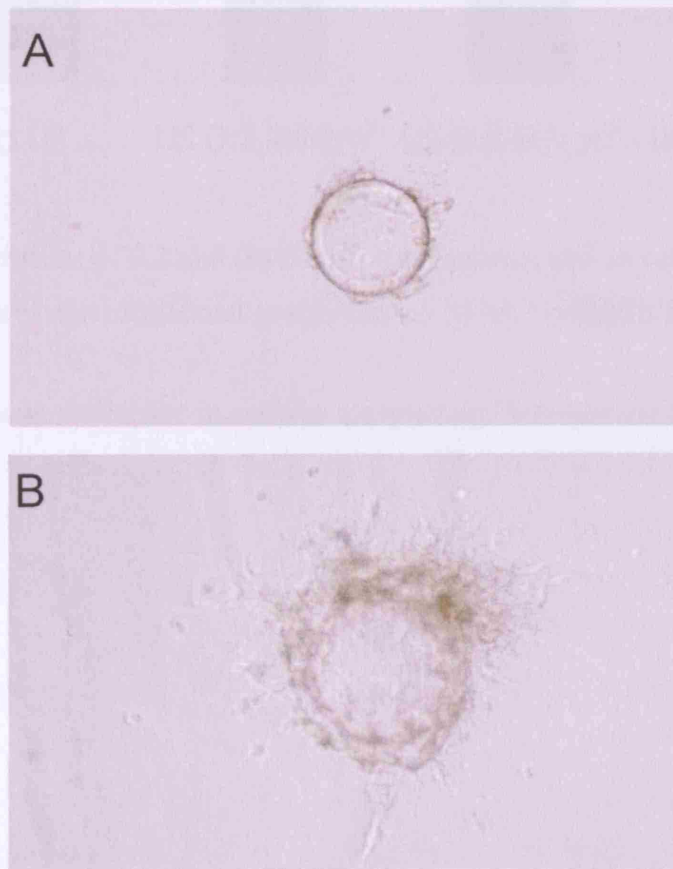
Figure 6.2

Endothelial cells showed no difference in morphology but clear differences in cellular migration between controls (A) and those treated with ultrasound (B)

The mean migration provided by US at 0.8 w/cm^2 was 3.17+/- 0.8 times greater than controls (figure 6.3). The greatest migration with Ultrasound in a single cell line was 3.95 greater than control gels. This migration equated to a maximum migration of 129 microns with a mean of 115.47 +/- 22.81.
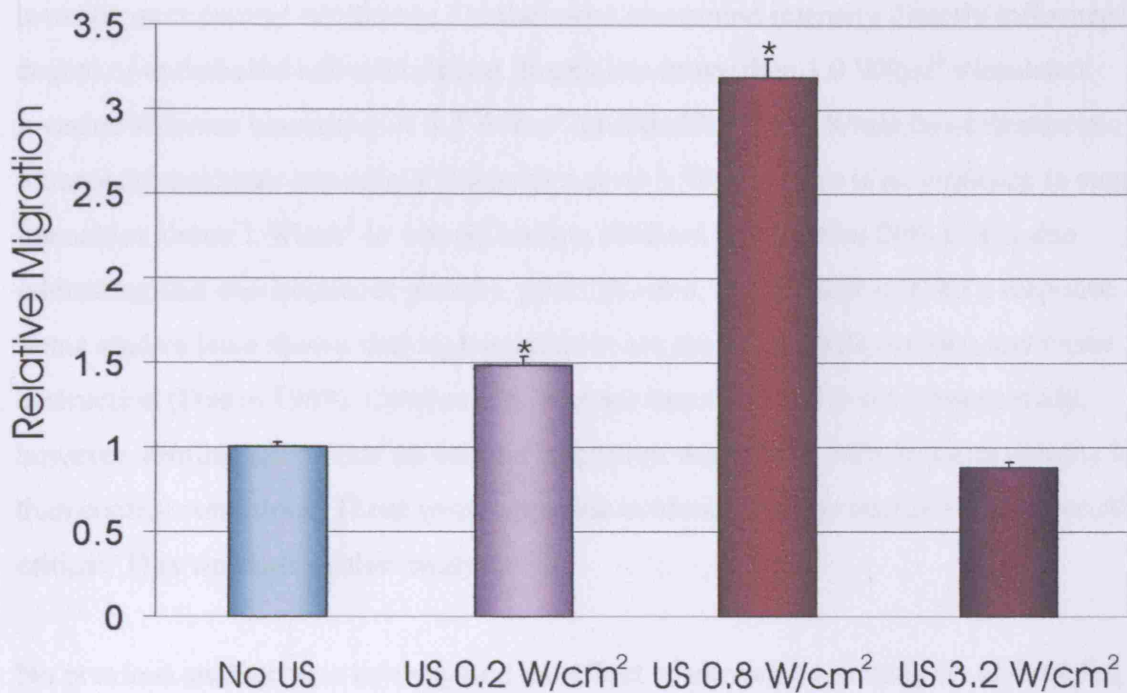
Figure 6.3 US intensities of 0.2 and 0.8W/cm^2 showed increases in cellular invasion relative to the control non ultrasound group (mn +/- SEM, *p<0.001, Dunn's, n=3)

There was no obvious difference in cellular morphology between the two groups (figure 6.2 A and B).

6.4 Discussion

These results show for the first time that ultrasound has a strong and direct effect on the behaviour of endothelial cells, promoting an invasive phenotype in collagen gels. Ultrasound, at its optimum intensity, provides a three fold increase in endothelial cell invasion over control conditions. Furthermore ultrasound intensity directly influenced the degree of endothelial cell stimulation. Intensities lower than 1.0 W/cm^2 stimulated invasion whereas intensities of 3.2 W/cm^2 inhibited invasion. While most therapeutic ultrasound machines can supply intensities up to 3 W/cm^2 there is no evidence to support intensities above 1 W/cm^2 in wound healing (Ballard and Charles 2001). It is also interesting that one treatment per day, given *in-vitro*, is sufficient to elicit a response. Some studies have shown that high intensities are associated with oedema and tissue destruction (Dyson 1968). Cytotoxicity was not measured but in the present study, however, diminished effects on cellular migration were clear, with invasive activity less than control conditions. These results provide evidence that the energy supplied could be critical. This warrants further study.

No previous studies have investigated the effect of ultrasound directly on endothelial cell functions *in-vitro* such as invasion. The result corroborates the single *in-vivo* rat wound model, showing a 20-30% increase in wound vascular density with US (Young 1990). The angiogenic response was thought to be mediated by cells such as leucocytes, lymphocytes and macrophages. Cellular effects were thought to be due to changes in cell membrane permeability (Ryaby, Bachner et al. 1989) most especially to calcium ion influx causing dramatic cell activation. Calcium ions act as secondary messengers which promote cell enzymatic activity and stimulate synthesis of specific proteins and their secretion (Katz and Repke 1966) . For example the formation or liberation of angiogenic factors FGF and TNFα has been observed. In this assay, the effect of US is more potent than the effect of any exogenous growth factor, on angiogenic ingress. Interestingly it is of the same magnitude as Fibrin. These results indicate that US stimulates endothelial cell invasion and an increase in activity within four days of plating in collagen gels. Further studies comparing the migration at four days and extended periods may add weight to Young and Dyson's observations that such a pro-angiogenic effect is short lived and does not continue beyond five days.

It is interesting to compare US with evaluations of other modes of therapy. While no studies have specifically observed endothelial cell invasion and migration, Tompach showed that Hyperbaric Oxygen stimulates endothelial cells to proliferate, with an increase in incorporation of radio labelled thymidine over controls. Hyperbaric Oxygen stimulates an 8-9 fold increase in vascular density over normobaric conditions (Marx 1990) and has been shown to increase the healing rate of chronic wounds by 68% over controls. While hyperbaric oxygen has an equal effect to bFGF on angiogenesis, the present results, that US stimulates endothelial cell invasion to 3 fold that of controls, exceeds the response to bFGF in the same assay. Heating, "warm up therapy", has been proven to stimulate chronic wound healing (Rothman 1954) by increasing the capillary density within wounds to 3 times that of controls. The similarity of response raises the question of whether the thermal effect of US may account for this.

This response, though encouraging, should be formally studied *in-vivo* to correlate to capillary density. The possibility that US stimulates the production of pro-angiogenic cytokines has been raised by previous studies. The availability of blocking antibodies or receptor inhibitors would allow investigation of these mechanisms.

6.5 Conclusions

This study has shown for the first time that ultrasound, administered using a clinical regime to endothelial cells in the *in-vitro* cytoball assay has stimulated endothelial cell migration 3 fold over control conditions. The intensity of US energy was shown to be critical. This chapter represents the first step in investigating the mechanisms of US action on endothelial cells.

Endothelial cell invasion is a key process in the formation of granulation tissue and the take of Synthetic Dermal Equivalents and skin grafts. If the results translate to *in-vivo*, in providing such a strong stimulus for endothelial cell invasion, ultrasound has the potential of being a valuable clinical tool in improving reliability of skin cover as well as stimulating wound healing. This response, comparable with the response to fibrin and FDPs in the same assay, was also comparatively greater than that of Topical Negative Pressure (chapter 7).

Much research has been conducted clinically into the effects of US on wound healing but there is no consensus over the optimum frequency, wavelength, intensity and pulse ratio for wound healing. This chapter has identified a window of US energy for optimum endothelial cell activation and demonstrates that higher energy suppresses migration. This represents a new therapeutic modality for US, which could aid wound healing and the take of bioengineered skin.

Chapter 7

In-vitro optimisation of Topical Negative Pressure regimes for angiogenesis into Synthetic Dermal Equivalents

7.1 Introduction

Chapters three to six have used a single *in-vitro* assay to explore mechanisms of increasing endothelial cell migration either through the matrix involved or the addition of growth factors or ultrasound to potentially increase SDE take. Take can also be increased by alternative mechanical adjuncts, the clinical use of which has increased with promising results. Some authors have used fibrin glue as an adhesive to successfully increase the take rate of dermal equivalents (Jeschke 2004). Studies have also advocated the use of Topical Negative Pressure (TNP) to stimulate dermal equivalent take, a treatment known to stimulate wound healing and decrease the depth of burn penetration. McEwan, using TNP at a constant pressure of 100mmHg, showed that negative pressure not only immobilised the graft, providing firm application to underlying tissue but also decreased fluid collection and consequently increased take (McEwan, Brown et al. 2004).

7.1.1 Topical Negative Pressure

Developed in the early 1990s by Argenta, Morykwas (Argenta and M.J. 1997) and Fleischmann (Fleischmann 1993), Topical Negative Pressure (TNP) applies a controlled force uniformly to the exposed tissues at the surface of a wound. The modality entails placing a sterile reticulated foam dressing (polyurethane ether 400-600 μm pore size or polyvinyl alcohol foam dressing) cut to the geometry of the wound which in turn is connected to an adjustable pump via a non collapsable tube. An adhesive occlusive dressing provides an air tight seal between the vacuum tube, foam dressing and surrounding skin, which allows suction to be applied by a vacuum pump. TNP has several recognised effects (figure 7.1 upper).

7.1.1.1 Mechanisms of action

1. Decreased interstitial fluid

Third space oedematous fluid mechanically compromises the microvasculature and lymphatic system (Argenta and M.J. 1997), which increases capillary and venous afterload. This occlusive pressure decreases the supply of oxygen, nutrients and growth factors to a wound. By occluding the lymphatic and venous system the removal of inhibitory factors (proteases such as cathepsins and elastases) and bacterial endotoxins is substantially decreased (Argenta and M.J.

1997). Macroscopic removal of the excess interstitial fluid (Isago 2003) following TNP decreases tissue turgor and diminishes capillary afterload, and thus increases local blood flow (Morykwas 1997) and wound perfusion.

2. Infection

By increasing local vascularity and tissue oxygen tension TNP decreases anaerobic bacterial colonisation (Argenta and M.J. 1997). Oxygen availability enhances neutrophil function and subsequent resistance to infection, through the oxidative burst mechanism, producing bactericidal oxidant radicals such as superoxide, hydroxyl radical and hyperchlorite (Hunt 1988). Morykwas has shown that within a porcine infected wound model (10^8 organisms/g tissue), TNP significantly increased the elimination rate of bacteria compared to control wounds (Morykwas 1997). Human trials emulate such results, with bacterial counts from wounds subjected to TNP decreasing from clinically significant levels to 10^2/g - 10^3/g tissue (Morykwas 1997).

3. Mechanical Stressors

Owing to the porosity of the reticulated foam dressing, TNP is able to apply a pulling/compressing force to the entire interface between sterile dressing and wound or SDE. The foam evenly distributes this centripetal force and pulls the edges together reducing cavity size. Generated mechanical loads permit vascularisation and tissue proliferation as shown with tissue expansion (Olenius, Dalsgaard et al. 1993) and the Ilizarov frame (Urschel, Scott et al. 1988). Tractional forces are known to stimulate cellular activity via transmembrane integrin adhesion complexes bound to the extra-cellular matrix, resulting in deformation of the cytoskeleton and release of secondary messengers (calcium ions, prostaglandins, inositol-triphosphate and phoshokinase C) (Ingber 1991; Ingber 1991). It has been hypothesised that application of TNP, by causing local external forces, can stimulate cells such as fibroblast and endothelial cells by these mechanisms, causing tissue budding/collagen production and angiogenesis respectively (Urschel, Scott et al. 1988; Morykwas 1997; Mullner 1997).

4. Angiogenesis, proteases and growth factors

The decompression of small blood vessels by third space fluid loss at a suction of 125 mm Hg promotes a four fold rise in blood flow (Morykwas 1997) and

stimulates arteriolar dilatation (Argenta and M.J. 1997). TNP acts both to stimulate blood flow and formation of new blood vessels from existing vessels (angiogenesis). Initial porcine studies conducted by Morykwas assessed subcutaneous tissue and muscle perfusion using Doppler probes in pig wounds with TNP (0-400mmHg) (Morykwas 1997).
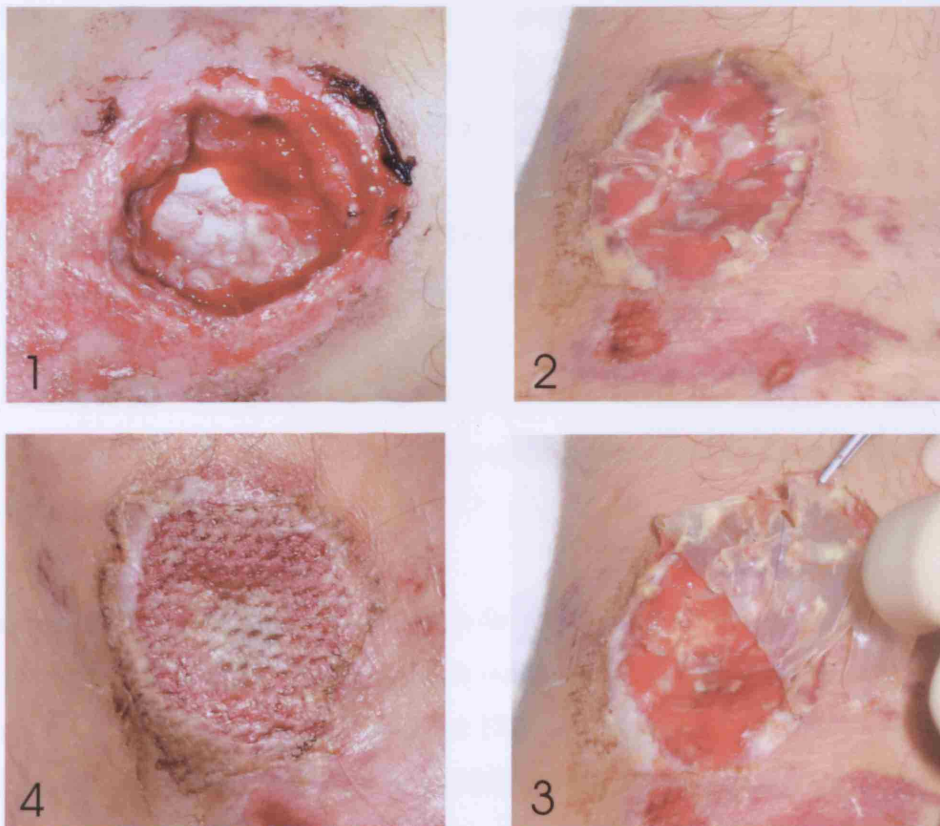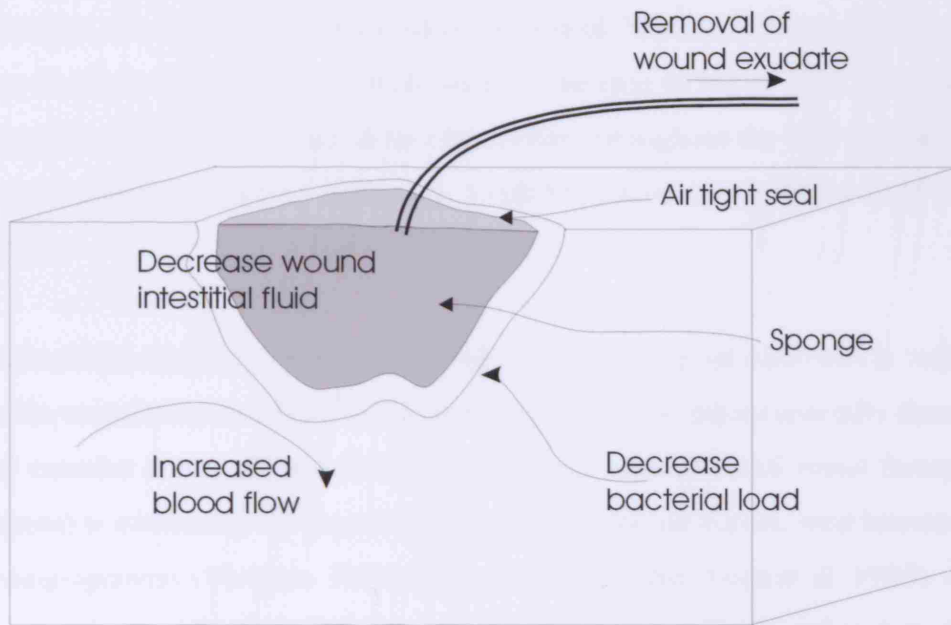

Figure 7.1 Mechanisms of action of Topical Negative Pressure (upper). Integra being used to cover open bone (lower 1), following 7 days of continuous Topical Negative

Pressure at 125mmHg (lower 2) the silicone membrane was removed (lower 3) and a meshed split skin graft (1: 1.5) was applied with concomitant TNP for one week (lower 4)

The optimum vacuum for granulation tissue formation was 125 mmHg provided it was applied intermittently (stopped for at least 2 minutes for every 7 minutes of therapy) otherwise this response was attenuated. The use of intermittent pressure has thus been advocated for all wounds for the first 48 hrs of TNP. Some authors consider that such a regime should be applied throughout the TNP treatment of pressure ulcers (Urschel, Scott et al. 1988; Morykwas 1997; Sibbald and Mahoney 2003).

These mechanisms should improve the overall rate of healing yet often this is limited by the available vasculature and thus TNP is not for all patients, most especially those with peripheral vascular disease. While this is true for large vessels, small vessel formation (angiogenesis) is stimulated by negative pressure. Mechanical forces, well known to stimulate angiogenesis (Virchow 1911; Cherry 1983; Urschel, Scott et al. 1988), promote secondary messenger release, cellular upregulation, matrix synthesis and endothelial cell proliferation. Capillary density has thus been found to be significantly higher within TNP wounds than controls (Ford 1989). This response is increased by intermittent application, causing repetitive secondary messenger release.

Wound fluid contains large quantities of proteases which inhibit wound healing (Xue, Le et al. 2006) and dermal equivalent take. This environment suppresses angiogenesis and keratinocyte, fibroblast and endothelial cell proliferation (Falanga 2002). TNP, by removing this protease-rich exudate, has been shown to decrease MMPs 1, 2 and13 and elastases (Clare 2002), and thereby moderates collagen and gelatin degradation, and acts to stimulate angiogenesis.

TNP's mechanisms of action clearly not only favour wound healing but also provide an optimal environment for graft take. Espensen commented on the effective take of Apligraf on diabetic foot wounds after initial TNP for 3-4 days at 100-125 mm Hg of continuous pressure (Clare 2002; Espensen 2002). This paper was a single case but considerable evidence confirms that TNP increases the take of SDEs, notably Integra. Molnar et al showed in a non-controlled study that 22 patients with exposed bone, joints or tendons achieved skin closure with Integra being applied with continuous negative

pressure for 8 days at 125 mm Hg with subsequent successful wound cover with split skin grafting with concurrent TNP for four days (Molnar 2004).

Optimum TNP regimes for wound healing have been provided by pre-clinical studies (Argenta and M.J. 1997) and subsequent evidence-based trials. However, the optimum regime for SDE take however has not been explored. Studies to date have used continuous pressure with pressures varying from 100-150 mmHg (Jeschke 2004; Molnar 2004). By designing an *in-vitro* TNP assay this chapter aims to establish:

1. The optimum regime of TNP to stimulate the migration of endothelial cells, a prerequisite for angiogenesis, and neovascularisation of SDEs.
2. The ideal macroscopic structure of a SDE to permit endothelial cell integration in conjunction with concomitant TNP.

The two leading SDE materials in the UK; Integra and Alloderm, have been compared in their ability to allow endothelial cell ingress against a cross-linked collagen control, Permacol, and a non cross-linked collagen control, Xenoderm.

Alloderm is decellularised non cross-linked human dermis which becomes vascularised and remodelled with host tissue after implantation (Wainwright 1996). The Alloderm processing removes the epidermis and the cellular components of the dermis, leaving the basement membrane components including type IV and VII collagen, laminin dermal collagen and glycosaminoglycans intact (Livesey 1995). The basement membrane surface promotes epithelial adherence (Ralston, Layton et al. 1999) and is thought to cause fewer adhesions and/or facilitate angiogenesis when used in dermal reconstruction.

However Alloderm, despite being comprised of dermal components, fails to integrate reliably with the wound, many studies stating take rates of 50%- 76% (Rennekampff, Pfau et al. 2002). Many have found that at least three weeks is needed for epithelialisation, with complete integration only at six months (Wagshall 2002). Ultimately, it results in a structured dermis with minimal scar tissue but often with an absence of rete ridges. Other studies have concluded that Alloderm provides a greater aesthetic result than split skin grafting, for this reason it has been adopted as an acceptable means of treating full thickness burns (Wainwright 1996), revising depressed

scars, nasal reconstruction (Silverstein 1997), facial defect repair (Achauer 1998), lip augmentation and septal perforation repair (Kridel 1998).

Integra is a two-layer reconstituted acellular foam matrix made from bovine collagen and chondroitin-6-sulphate, cross-linked with glutaraldehyde. The upper silastic sheet is an epidermis like structure which controls water loss and prevents the invasion of microbes. The lower layer has a porous structure, is composed of a cross linked co-precipitate of collagen and chondroitin-6-sulphate and functions mainly as a template for dermal regeneration. The dermal template becomes revascularised within 21 days after grafting and must then be covered with an epidermal graft. The resulting coverage is thus pliable and is not adherent to deeper structures (Dantzer and Braye 2001). Several studies demonstrate good aesthetic and functional results in burn treatment and reconstruction with Integra; however those studies have also delineated the problems of Integra's unreliable take rate and the long intervals needed between surgical intervention (Moiemen 2001).

To tissue-engineer a viable dermis, it is important that cells migrate into the ECM scaffold, proliferate and organise the architecture of de-novo tissue and synthesised matrix. Current literature does not indicate the propensity of the afore-mentioned matrices to support cellular infiltration and therefore such a characterisation may advance the science of dermal tissue engineering.

The aims of this chapter are to compare and contrast the angiogenic potential of these SDEs and evaluate the potential of their matrix structures to allow endothelial cell ingress. It also aims to establish, by using a novel *in-vitro* vacuum pump, an optimum regime of Topical Negative Pressure to increase this ingress and thus potential SDE take.

7.2 Materials methods

7.2.1 In vitro assay of Topical Negative Pressure

Polycarbonate chambers with interposing Polytetrafluoroethylene washers, (depth of 2mm and 1mm) were manufactured and assembled as previously outlined (chapter 2.15, figures 7.2, 7.3).
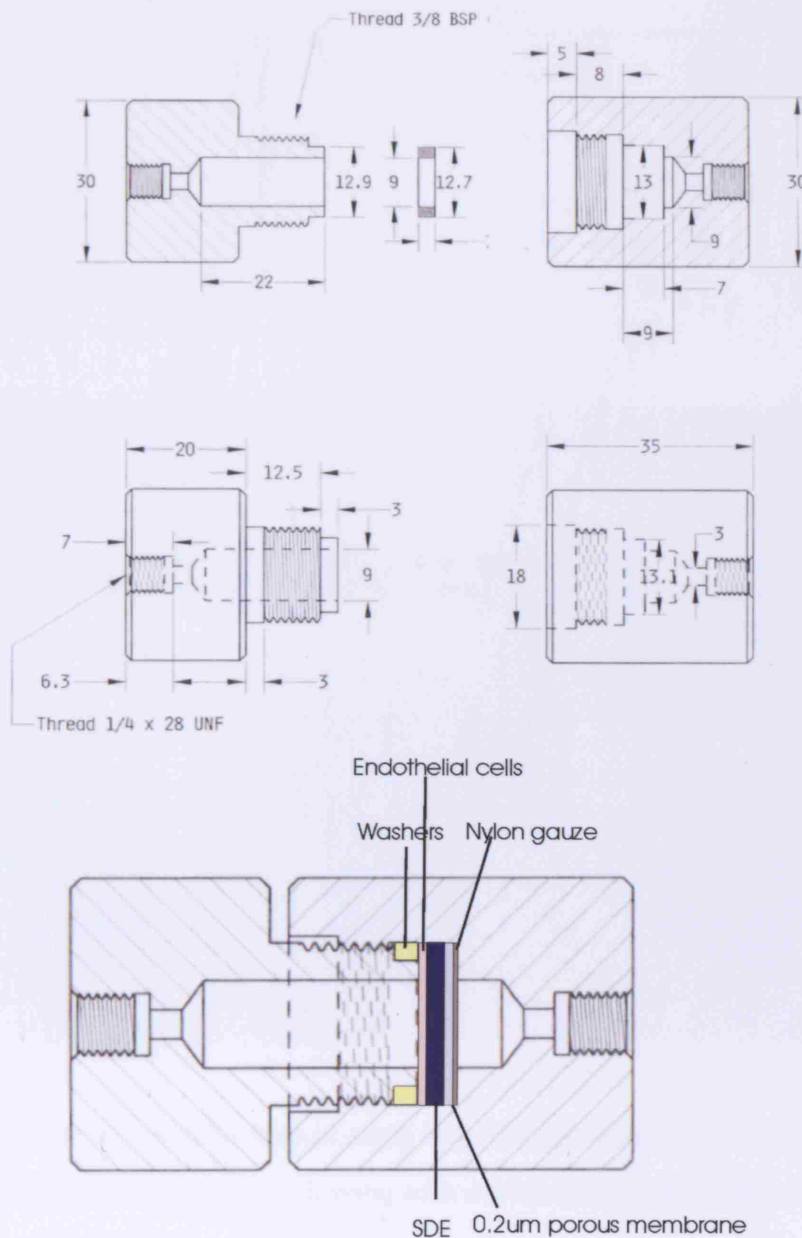
Figure 7.2 *In-vitro* Topical Negative Pressure chambers were made in two sections from Polycarbonate (male and female 3/8 BSP thread), fitted with luer-lock ¼ 28 UNF thread connectors and assembled with interposing PTFE washers, membranes and gauzes (lower). Dimensions in mm.

7.2.2. In-vitro assay

Three different endothelial cell isolates were harvested from established confluent cultures (chapter 2.2.3) and added drop-wise to the assembled TNP pumps once the respective SDE was aseptically placed in the chamber. The chambers, containing air, media, cells and Synthetic Dermal Equivalent were incubated at 37 C in 5% CO_2 for 24 hours.
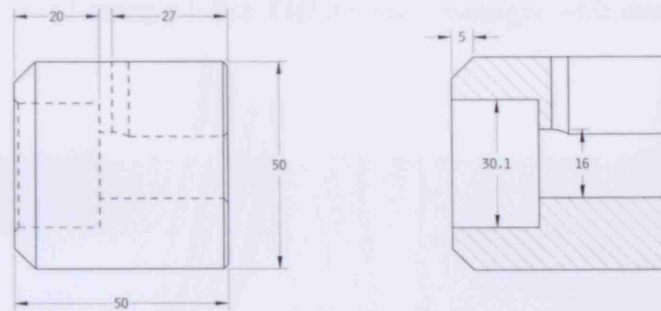
Figure 7.3 Bases for the chambers were made from black Delrin (upper), the *in-vitro* chambers were supported on bases allowing tube access to the underside luer port (lower). Dimensions in mm.

At 24 hours all chambers were removed from the incubator and attached using the Luer lock system to two negative pressure pumps, a KCI pump giving intermittent pressure and a Welsch pump giving continuous negative pressure. SDEs were further incubated for 48 hours with or without the negative pressure. After two days the SDEs were fixed,

blocked, sectioned and stained histologicaly (chapter 2.8) and assessed for cellular migration using digital image analysis.

7.2.5 Image analysis

Images were taken using a DC200 camera on an inverse Nikon Eclipse TS100 light microscope or a Zeiss Axioskop inverse phase light microscope at 10 x magnification. Images were recorded using a Leica IM50 image manager with image size 1280 x 1024 (Figure 7.4).
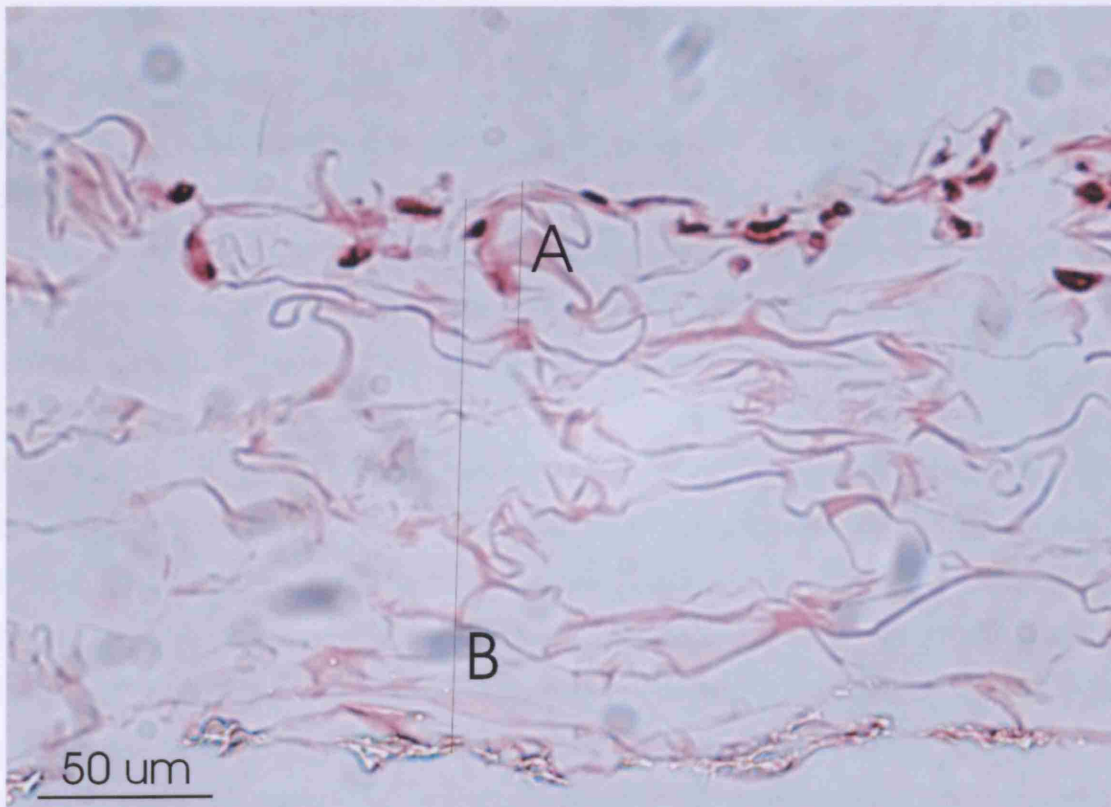
Figure 7.4 Following incubation of the SDEs with endothelial cells and media, the SDEs were fixed and sections were taken for histology. Following staining, cellular migration was measured as a fraction of the depth of the matrix (A/B x 100).

7.3 Results

7.3.1 Use of the in-vitro TNP pump

Initial pilot studies using the apparatus without any supporting Nylon gauze resulted in tearing of the $0.2\mu m$ pore membrane on application of the vacuum and established the need for a physically tough supporting base layer. Shrinkage of SDEs and leakage of media was also associated with vacuum application. This was overcome by using two PTFE washers with the thinner SDEs (Xenoderm) and regular tightening of the two chamber sections.

7.3.2 Endothelial cell invasion

The direct measure of mean endothelial cell invasion distance through a SDE gave similar results as the fractional depth of SDE parameter (figure 7.6).

Cellular morphology observed on histopathological slides varied between the matrices. Cells were spread with clear visibility of the cytoplasm in Integra, whereas on the collagen controls they were more rounded and clumped (figure 7.5).

7.3.3 Integra provides the best environment for the migration of endothelial cells

In all conditions with all SDEs, cells were seen at the top layer of the matrix following 48hrs of incubation with or without TNP. Endothelial cells invaded a greater fractional depth of Integra than any other SDE in the 48 hours of incubation. In both of the collagen controls, Permacol and Xenoderm, migration of cells was minimal. Integra and Alloderm gave greater endothelial cell migration over the collagen controls ($p<0.05$ Tukey, $n=3$) but Integra gave greater migration than Alloderm in all conditions ($p<0.05$ Tukey $n=3$, figure 6.6).

7.3.4 TNP increased endothelial cell migration through SDEs compared to control conditions

The application of Topical Negative Pressure increased endothelial cell migration. This response varied according to the TNP regime and the SDE. TNP had no influence over endothelial cell migration in the collagen controls ($P>0.05$ Tukey, $n=3$, figure 6.6). With Integra, intermittent TNP provided a statistically increased endothelial cell migration over any alternative TNP regime, however no difference was found between 4hrs of TNP and no TNP. A similar response was seen with Alloderm with intermittent providing by far the greatest invasive response.
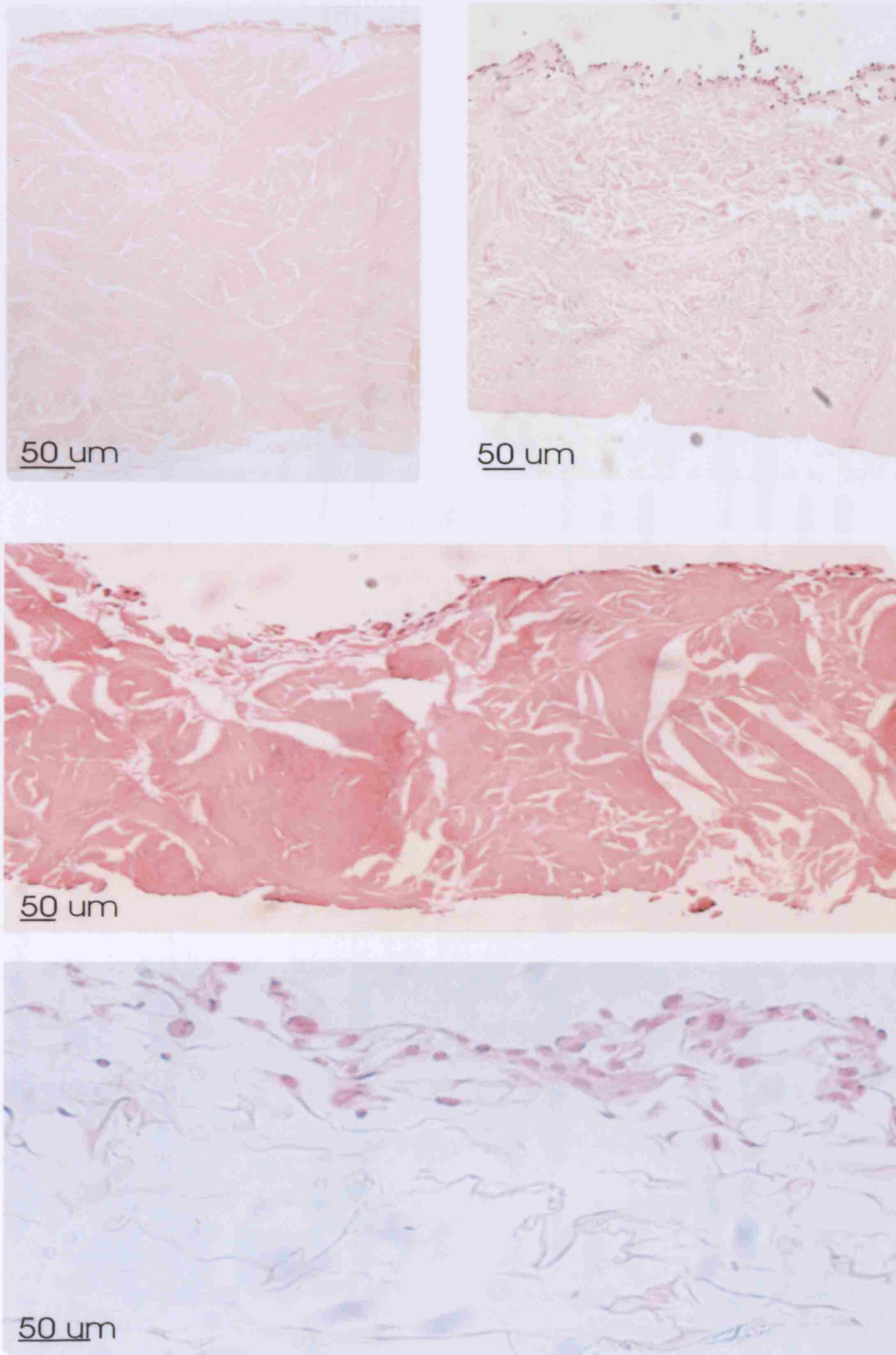
Figure 7.5 Histological sections of the four SDEs following a 4 hourly TNP regime (Permacol upper left, Alloderm upper right, Xenoderm middle, Integra bottom) were all measured and compared for endothelial cell migration.

7.3.5 Cross-linking has no effect on endothelial cell invasion

Cross-linking of the SDE had no influence on endothelial cell invasive phenotype independent of the TNP regime used.
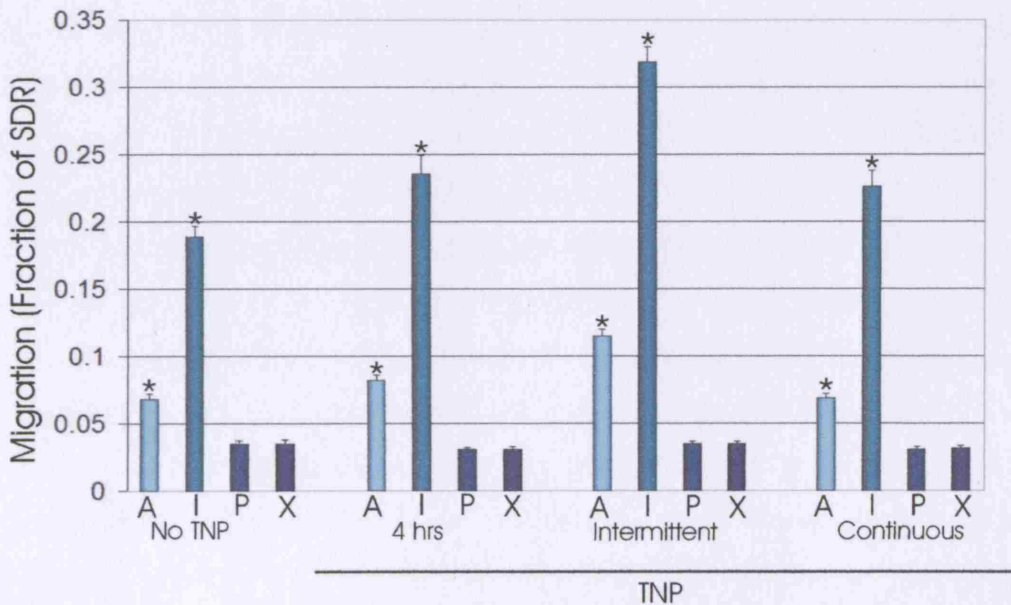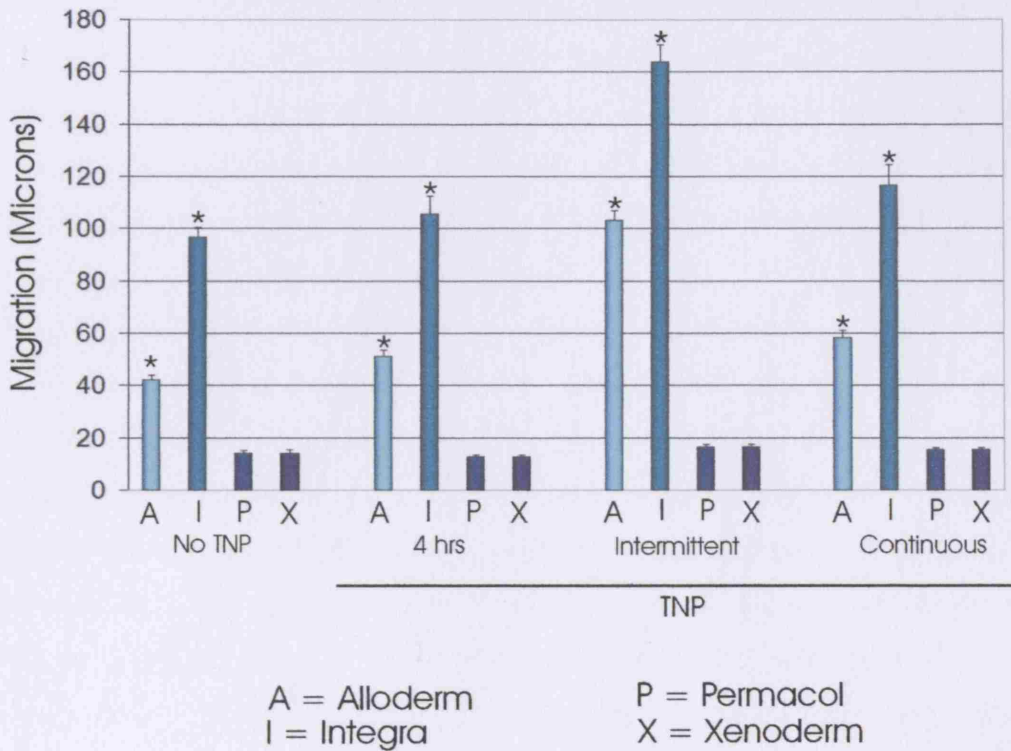
Figure 7.6 Each SDE was assessed for mean distance of endothelial cell migration through the matrix with or without the addition of three regimes of Topical Negative Pressure (4 hours per day, intermittent 5 minutes on 2 minutes off, continuous, upper).

Distance of migration was also calculated as a ratio of the depth of each matrix (lower, mn +/- SEM, * p < 0.05, n=3).

7.4 Discussion

In order to understand the angiogenic potential of Topical Negative Pressure and it's recently published ability to increase the take of Synthetic Dermal Equivalents, this chapter has taken an *in-vitro* approach to measure endothelial cell migration into different SDEs using a novel chamber. Comparisons of angiogenic potential of SDEs have not been made previously with or without Topical Negative Pressure. Although TNP at an optimum pressure of 125mmHg has been found to stimulate blood vessel formation, the regime for angiogenic ingress into SDEs has not been previously investigated.

The *in-vitro* chamber used showed that, of the SDEs studied, Integra supports the greatest basal invasive response of endothelial cells, 10 times that of Permacol or Xenoderm and 2.5 times that of Alloderm. No other study has specifically looked at endothelial cell migration through such a matrix. Similar studies without using negative pressure (Jones 2003) (using Keratinocytes) have shown Integra to be 'pro-migratory,' although this was defined in terms of allowing migration through the whole thickness of Integra in 14 days (20-30 µm/day). While the *in-vitro* assay does not measure the complete endothelial cell transit time of the SDE, it showed that the open collagen/chondroitin-6-sulphate matrix allows endothelial cell penetration to 100 µm (0.189 of its depth) in 48 hours without negative pressure (50 µm /day). This pro-angiogenic response concurs with the *in-vivo* findings with a similar atelocollagen sponge, in which vascular invasion has been seen at 7 days in human studies (Molnar 2004). The results here contrast with human studies in which Integra, used as a reconstructive option in a wide array of clinical indications, took up to 14 days to promote endothelial cell migration (Moiemen 2001). However, cell invasion measured in these studies does not equate to angiogenesis *in-vivo*, but may be a useful comparative indicator of angiogenic potential of a SDE. The addition of negative pressure to Integra greatly stimulated an increase in migration to 160 µm (0.318 of its depth) in 48 hours with an optimum intermittent vacuum regime (80 µm/day). Though the data in this chapter only measures the degree of endothelial cell invasion in 2 days, this maximal effect of intermittent TNP could translate to a 60% reduction in time for take. This compares well with the clinical data on complete revascularisation times (mean 7.25 days) seen with a continuous TNP regime with Integra on exposed bone and soft tissue (Molnar 2004).

7.4.1 Alloderm/Integra

Here it was shown that Alloderm, an acellular non cross-linked dermal replacement, gave less endothelial cell invasion under basal conditions than Integra but more than collagen controls. Alloderm's structure is denser than the Integra yet more open than the collagen controls. This *in-vitro* data corresponds well with clinical studies showing that Alloderm takes 3 to 4 times longer than autografts to take (Wax, Winslow et al. 2002). Nevertheless, the degree of endothelial cell ingress into Alloderm concurs with *in-vivo* and clinical studies in which histological analysis of Alloderm grafting confirms its potential for complete vascularisation (Menon 2003). While not looking specifically at endothelial cells, other *in-vitro* studies have commented on the propensity of Alloderm to support cell proliferation yet limit cellular integration and migration (Ng, Khor et al. 2004). This was shown to a degree in our study when compared to Integra (figure 7.5). The difference in infiltration between Integra and Alloderm may be due to the macroscopic structure of the matrix, the composition, or both. The open foam structure of Integra would logically seem to aid cellular migration and revascularisation, although its pore size, being far greater than cellular dimensions, may be unnecessarily large. Although Alloderm is much denser than Integra, the endothelial invasion it supports is 40% that of Integra and 3 times that of Permacol or Xenoderm, even though it is only slightly less dense than these latter equivalents. Alloderm consists of acellularised dermis and will include natural glycosaminoglycans. Integra also contains Chondroitin-6-sulphate a glycosaminoglycan that is known to induce a pro-angiogenic endothelial cell phenotype in matrices (Tapon-Bretaudiere 2002; Potter, Cussons et al. 2006) and as such may contribute to the pro-angiogenic effect of Integra.

7.4.2 Permacol/Xenoderm

Permacol is porcine collagen cross linked with hexamethylene diisocyanate, it is widely used in the clinical setting as a means of treating skin contour irregularities (Saray 2003), reconstructing anatomical structures such as the orbital (Cheung, Brown et al. 2004) and pelvic floor (Cosson 2003) and more recently has been marketed and used as a dermal replacement (MacLeod, Sarathchandra et al. 2004). While some studies have shown that Permacol successfully vascularises *in-vivo* after 2 weeks and subsequently is able to support autologous grafting, these results show that this matrix resists endothelial cell

invasion with or without a negative pressure environment. Our data compares well with others that have observed resistance to cellular (fibroblast) invasion and a propensity for fibroblasts covering the matrix to stack up on each other rather than migrate (Jarman-Smith 2004) (compare with endothelial cells in figure 7.5). Many units have seen, not only poor clinical biocompatibility (MacLeod, Sarathchandra et al. 2004), but an increase in: inflammation, pain and a foreign body type reaction to the matrix (Belcher and Zic 2001). Some advocate that this response is due to cross-linking making the matrix resistant to collagenases (Jarman-Smith 2004), impeding cellular integration. However, Xenoderm in this chapter gave no statistical difference in migration from Permacol despite the fact that it is not cross-linked. Other cross-linked matrices support faster clinical take and far greater *in-vitro* cellular migration.

A principle contributor to the pro-angiogenic effects of Integra and Alloderm is likely to be the porosity of the structures as compared to Permacol and Xenoderm (figure 7.5). It is well known that the potential of some biocompatible scaffolds for tissue engineering is critically influenced by the pore size which in turn determines angiogenesis and reduces foreign body reactions (Pieper 2000). Direct comparisons of matrices of identical components, yet different macrostructures, would clarify the importance of structural variables. These results from four predominantly collagen-based SDEs demonstrate that physical characteristics such as porosity are of prime importance in determining the success of integration of a dermal matrix material.

An intermittent regime of TNP over the 48 hour test period provided greater endothelial cell migration than continuous, 4 hour only, or control regimes, this was marked with Integra. Whilst no other study has directly looked at endothelial cell migration in response to negative pressure, initial work by Argenta and Morykwas (Morykwas 1997) showed that the optimum regime for granulation tissue formation was intermittent rather than continuous. Studies that have explored the role of TNP in increasing the take of SDEs are often confined to small series or case reports and, despite finding encouraging results, have not clinically compared the pressure regimes available to investigate the different take rates with different pressure parameters. In using an *in-vitro* device this study has allowed the direct comparison of TNP regimes. It has provided evidence that an intermittent regime statistically increases, by a factor of up to 60%, the rate at which endothelial cells invade a matrix. This suggests a mechanism to accelerate revascularisation and graft take.

7.5 Conclusions

In using an *in-vitro* model of angiogenesis that can be manipulated to apply controlled Topical Negative Pressure this work has given evidence that Integra, of the four SDEs tested, has the greatest potential for endothelial cell invasion. These results suggest that the physical structure and porosity of a SDE could be a principle determinant of angiogenic potential. It has also been shown that intermittent application of this suction optimises endothelial cell invasion, suggesting this modality could increase graft angiogenesis and take. Further avenues for investigation using this *in-vitro* approach include exploring the viability of the cells after TNP and whether there are differences in cellular survival between the regimes. Clinical studies exploring the angiogenic response with SDE grafting and TNP regimes should now be considered to corroborate such encouraging *in-vitro* data.

The majority of studies investigating SDE take with TNP have used a continuous regime with encouraging results. These *in-vitro* results suggest potential for a beneficial change in clinical practice.

Chapter 8

General Discussion

Despite advances in the surgical treatment of small wounds with the advent of micro-surgery and advances in understanding of fasciocutaneous and musculocutaneous vascular anatomy, the treatments of large wounds such as burns remains a problem and as such is associated with considerable morbidity and mortality. Advances over the last 30 years have given cultured keratinocytes and Synthetic Dermal Equivalents to recreate the bilayer skin. These however remain hindered by poor take rates and thus provide an ineffective yet expensive treatment.

This thesis has aimed to provide the research behind the formation of a second generation Synthetic Dermal Equivalent by targeting angiogenesis, a processes deficient in present day SDEs.

Cultured endothelial cells were assessed for their ability to migrate through ECM gels and SDEs. Two *in-vitro* assays were used: a multi well assay used to assess migration through ECMs with or without growth factor stimulation or ultrasound and a TNP chamber model allowing the measurement of endothelial cell migration in different SDEs with or without concomitant negative pressure. The use of a single assay allowed comparisons between ECMs and growth factors to give the optimum environment/matrix for potential use in a second generation SDE. The TNP *in-vitro* assay allowed comparisons of SDE macro structure as well as optimum negative pressure regimes for endothelial cell migration.

8.1 Clinical implications

8.1.1 The performance of Extracellular Matrices

In assessing and comparing the performance of extracellular matrices in allowing endothelial cell invasion chapter three has shown that despite collagen being the principal component of SDEs available commercially, it failed to provide a pro-migratory matrix for endothelial cells. It was out-classed by most of the glycosaminoglycans as well as vitronectin and fibronectin. It provided nearly seven times less migration than fibrin. Nevertheless as a viable scaffold it provided a solid foundation for endothelial cell migration (chapter 7). Fibrin was found to induce the greatest endothelial migratory respose and thus likely potential for revascularisation of dermal equivalents (chapter 3).

This thesis found that fibrin degradation products provided an equal stimulus for endothelial cell migration to fibrin within a collagen environment. This was only true for fibrin degradation product E (FnE). Fibrin degradation product D (FnD) did not provide as great a stimulus as fibrin or FnE. FnE is a soluble protein, this is mechanistically interesting in suggesting that;

1. soluble molecules can "reproduce" the whole fibrin effect
2. fibrin can function as a reservoir of soluble factors

Thus FnE could be incorporated into a parent SDE structure in order to provide equal endothelial cell migration to fibrin.

8.1.2 To assess the stimulus of growth factors in increasing endothelial cell invasion through extracellular matrices

Chapter 4 has shown that growth factors do increase endothelial cell invasion through collagen gels and that this is dependent on growth factor concentration and this varies according to the agent used however with little variation between the factors. Though much research has concentrated on such factors in mediating an increase in angiogenesis into matrices, these factors, at their optimum concentration, did not provide the same degree of migration as fibrin. In a fibrin matrix the addition of VEGF actually impeded endothelial cell migration. The effect of growth factors was modest if at all. This thesis has provided little support for growth factor use within a future SDE to improve endothelial cell migration and thus take.

8.1.3 To study the effects of ultrasound on endothelial cell invasion *in-vitro* as a potential therapeutic avenue for increasing SDE take.

The use of ultrasound is well documented in aiding wound healing. Little research has investigated Ultrasound in stimulating the take of SDEs or skin grafts, however this chapter provides clear data that Ultrasound can directly stimulate endothelial cell migration and as such warrants further *in-vivo* investigation. It is suprising that Ultrasound provided significant migration above that of the growth factors yet the use of the latter has had significantly more research in wound healing, flap viability and graft

take than the former. The research also showed that a therapeutic window exists and that high energy is deleterious. Hence therapeutic use may need careful monitoring. BecauseTNP was assessed with a different *in-vitro* assay, comparisons between US and TNP cannot be drawn, nevertheless significantly more research has been spent on negative pressure than US.

8.1.4 To study the effects and compare regimes of TNP in increasing endothelial cell invasion into SDEs (chapter 7)

Negative pressure stimulated an increase in endothelial cell migration through the SDEs tested. Nevertheless this chapter refutes a hypothesis that the greatest response would be from a continuous application of the pressure. Surprisingly an intermittent regime stimulated the greatest migration. Clinical applications of TNP to increase SDE take mostly has used a continuous regime. No clinical study has compared TNP regimes with SDE take and thus this chapter has immediate implications in clinical practice.

Chapter 7 studied the effects of TNP on four matrices, two with a relatively open type matrix scaffold (Integra and Alloderm), the other two (Xenoderm and Permacol) with a comparatively dense structure. Both Integra and Alloderm allowed greater endothelial cell migration, Integra having the greatest migration and also having the most open structure.

The potential of some biocompatible scaffolds for tissue engineering is critically influenced by the pore size which in turn determines angiogenesis and reduces foreign body reactions (Pieper 2000). To directly compare structures and porosity with identical protein composition was beyond the scope of this thesis but would clarify the optimum composition or porosity that would provide the greatest migration and thus potential integration. These results, from four predominantly collagen-based SDEs, demonstrate that physical characteristics such as porosity are of prime importance in determining the success of integration of a dermal matrix material.

8.2 Critique

The cytoball assay is a validated assay that has been used successfully by other authors. In this study it was used purely as a means of assessing endothelial cell invasion into

matrices. While endothelial cell invasion is a key process in angiogenesis there are two other processes mainly proliferation and tubulogenesis. One could intimate, from looking at the images from those conditions in which prolific migration was seen, that the cell clearly had increased in number from the limited number attached to the cytoball on placing into the matrix. Nevertheless this was not objectively assessed. There would be greater clarity if all conditions had been assessed for tubulogenesis and proliferation as well as migration.

Many of the matrices and growth factors used gave a direct effect or an inverse effect of migration to concentration. Others gave a biphasic response. The agents tested in the cytoball assay were mostly at three different concentrations. These concentrations were thought to span their optimum, as determined by like studies. The limited number and range of concentrations used per agent could induce error in that a rise in migration in line with concentration could be the beginning of a biphasic response. Likewise a diminishing response with an increase in concentration could be the end of a biphasic response curve. While the concentrations used in these studies relate to published response data, a greater range of concentrations would have extended the scope of the results.

Chapter 7 used Topical Negative Pressure to ascertain the degree of migration over 48 hours of endothelial cells into SDEs. This gives an indication of the angiogenicity of the matrices involved. It would be useful to investigate the length of time needed to populate the full thickness of the graft and thus an indication of the length of time needed prior to grafting of the SDEs with either split skin or cultured keratinocytes. With the cytoball assay one could directly visualise cellular movement from day to day and get a degree of viability of the endothelial cells and this was not possible with the TNP assay. Assessment of cellular viability prior to fixation at the conclusion of the experiments would have determined whether cells actually survived the assay corroborating the histological appearances which did not suggest that cell death was a major occurance.

Endothelial cells migrated through the matrices in response to control or negative pressure conditions. The increase in travel of the cells in response to the pressure was thought to be due to a change in cellular phenotype. This chapter did not address the question that the cellular movement may not have been due to migration per se but a mechanical pull on the cells through the matrix and thus the more open the matrix the greater the response.

Further studies into the effects of ultrasound, dose duration compared with the dose energy and the number of treatments (dose schedule) per day would have extended the power of the study

8.3 Conclusions

- TGFβ of the nine growth factors tested gave the greatest endothelial cell migration. This was far less than fibrin.

- Collagen had a biphasic concentration endothelial cell migration response.

- Endothelial cell migration in collagen gels was superseded by the addition of any of the nine extracellular matrices tested.

- Fibrin, when added to collagen, promoted the greatest endothelial cell migration of the tested ECMs, this being 3.4 times that of collagen alone.

- Fibrin alone promoted well over six times the migration of collagen alone.

- The addition of further ECMs to a collagen fibrin matrix inhibited migration.

- Growth factors stimulated an increase in endothelial cell migration by only 1.15 – 1.98 over control conditions.

- Both fibrinogen and thrombin induced an invasive endothelial cell phenotype to equal or greater effect than the growth factors.

- Fibrin imparted a pro-angiogenic effect by inducing an autocrine VEGF release from the endothelial cells.

- Both Fibrin degradation products D and E were pro-migratory to the endothelial cells. E imparted greater migration than D, the former being equal in effect to fibrin.

- Ultrasound induced an increase in endothelial cell migration in collagen gels. This response being greatest with 0.8 W/cm^2 intensity

- Topical Negative Pressure increased the migration of endothelial cells through SDEs. An intermittent regime giving greater migratory response than continuous.

- Integra supported greater endothelial cell migration than Permacol, Alloderm or Xenoderm, with or without concomitant Topical Negative Pressure.

8.4 Proposals for further studies

This thesis has outlined potential components for inclusion within a second generation Synthetic Dermal Equivalent. In certain areas, most especially fibrin, it has further investigated the mechanisms by which these molecules impart their effect. The thesis has also investigated the potential of TNP and Ultrasound as external mechanisms of increasing endothelial cell ingress into these matrices. It has analysed the effects of the structure of the equivalent and found that a loose foam matrix potentially promotes greater migration than a cadaveric matrix of densly packed collagen. Further aspects of matrix synthesis and viability need to be explored in order to manufacture a second generation pro-angiogenic matrix. While this thesis has found a foam to be comparatively pro-migratory, matrices can be manufactured from woven fibres as well as sheets. Components, as shown in chapter seven, can be cross-linked. Numerous agents can be used for this process none of which have been thoroughly investigated for their effects on angiogenesis.

This work will therefore continue as a long term project in providing a viable second generation pro-angiogenic SDE. In the short term it will achieve this by testing ECM components in different physical forms. Matrices will be made using foam, by a well established method. Fibrous matrices will also be made using an extrusion/curing process. The foam method has been well established for dermal matrices, essentially, the matrix is generally suitable for matrix proteins or protein/glycosaminoglycan mixtures, it has been used for lab scale preparation of dermal equivalents (Integra). Extrusion was developed for fibronectin/fibrinogen cryoprecipitates as a method for manufacturing ECM fibres of controlled diameters (e.g. 200μm) and with a tensile strength suitable for tissue repair. The method is versatile for composition of different matrix proteins.

A super-concentrated ECM protein precipitate/dope mixture is prepared, which is formed into a fibre of aligned molecules by extrusion through a fine cannula. The fibre is collected onto a rotating drum, and can be formed into a criss-cross lattice by sweeping the fibre across the drum. The extruded fibres are cured in a reagent bath. This stage can be varied with different acidic or other reagents to precipitate and cross-link the fibre proteins. This method is currently being used in the RAFT laboratories for tendon repair research, and will be readily accessible for this project.

Dermal equivalents will be prepared incorporating pro-angiogenic fragments into a standard matrix formulation. Both the foam and fibre methods will be used for collagen and fibrin based matrices. Matrices will undergo stability studies with various proteases noting half-life and the effects of cross-linking with glutaminase, glutaraldehyde and dicarboimide. Smart matrices will be compared to control matrices such as Integra™ and Permacol™.

Another aspect of the development of bioartificial skin is the transfer of technologies from laboratory to manufacture. Issues about transmission of prions and viruses in pooled human or animal products, as well as production consistency have proved to be obstacles to industrial scale production. The smart matrix will be developed in consultation with Professor Peter Dunnill, Dept Biochemical Engineering, UCL. One option is to base the final matrix on synthetic peptides linked to an inert biodegradable matrix, this could be expensive as a product. A favoured alternative is microscale production of autologous biomaterial (e.g. fibrinogen/fibronectin fraction).

8.4.1 Validation of matrix

A dorsal skin fold chamber model will be used to compare the *in-vivo* angiogenic potential of novel matrices produced by either foam or fibre extrusion. These will be assessed against clinically used alternatives (Integra). This *in-vivo* assay allows the non-invasive observation and quantification of angiogenesis in mice skin, using still or video microscopy , allowing each mouse to be followed over time. Synthetic Dermal Equivalents implanted in observation windows made in the dorsum of mice are assessed every 3-4 days, up to 2 months (using laser Doppler), for the physiological properties of blood vessels; vascular density, vascular diameter and RBC velocity.

Dermal matrices can be held under a measuring grid. Vessel diameter and RBC velocity could be periodically confirmed using Fluorescein Isothiocyanate (FITC) labelled dextran. Functional vascular density can be estimated by the total length of perfused microvessels per unit area of observation. Dermal differentiation, vascularisation and viability would be histologically assessed (routine Haematoxylin & Eosin, and endothelial cell staining for PECAM) at 2 months. The skin equivalents to be assessed

include collagen + chondroitin 6 sulphate, collagen+ fibrin subunit E, collagen + proangiogenic subunits, and Integra™.

The above outline is a short term plan and eventual pro-angiogenic matrices should be tested in porcine wounds before progressing to a clinical trial.

Ultrasound while stimulating endothelial cell migration may likewise have affects on cellular viability and also should be considered in the cytoball assay. While chapter 6 concentrated on the effects of altering ultrasound intensity and endothelial cell invasion further work should also be done on the effects of pulsatility and frequency.

As stated in the critique the *in-vitro* TNP study should continue assessing cell viability, the cause of the movement of the cells be it either a change to a migratory phenotype or more a mechanical pull. The time course of this *in-vitro* work should also be increased to investigate time to full thickness migration of each SDE and thus the potential time for "epidermal" grafting. Proliferation rates and tubulogenesis with in the matrices in response to TNP should also be considered.

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Prizes/ Grants/Awards

1) Hunterian Professor/Lecturer, 2005/6. Awarded by The Royal College of Surgeons of England for the lecture entitled "Transforming the treatment of burns with a proangiogenic skin substitute, a new paradigm in the management of large wounds."

2) Patrick MacNamee prize. April 2004, RAFT. Awarded at the RAFT annual seminar in open competition for the presentation judged by external peer review to be that which best epitomises the founding principles of RAFT, namely the combination of clinical relevance and scientific research.

3) Stanley Thomas Johnson Foundation Project Grant. March 2004. The Stanley Thomas Johnson Foundation. A peer review grant from an international charity based in Switzerland.

4) Dunhill Medical grant, 2002. Dunhill Medical trust. A grant awarded for research into wound healing.

5) Alan Gaynor Fellowship, 2002. Alan Gaynor Foundation. A grant awarded for research into burns and scarring.

Publications

1. Potter M. Linge C, Dye JF, Sanders R. An investigation to optimise angiogenesis within potential skin equivalents. Plastic and Reconstructive Surgery, Plast Reconstr Surg. 2006; 117(6):1876-85.

2. Potter M, Banwell P, Baldwin C, Elizabeth Clayton E, Irvine L, Linge C, Grobbelaar A, Sanders R, Dye J, *In-vitro* optimisation of Topical Negative Pressure regimes for angiogenesis into Synthetic Dermal Replacements. Burns. In press, 2007.

3. M Potter, C Linge, R Sanders and J Dye, Fibrin as a Potent Stimulus of Endothelial Migration, in 23rd European Conference on Microcirculation (Lisbon, Portugal, September 8-10, 2004), eds Martins e Silva J., Saldanha C., Oliveira V., Prie A., Shore A., Bologna, Medimond, 2004, ISBN 88-758-7078-0, 131-135.

4. Petrie N, Potter M, Banwell P. The management of lower extremity wounds using topical negative pressure. International Journal of Lower Extremity Wounds. 2003; 2 (4): 198-206.

Presentations

1. Potter M, Linge C, Cussons P, Dye J, Sanders R. Transforming the treatment of burns with a proangiogenic skin substitute, a new paradigm in the management of large wounds. Hunterian Lecture, Winter BAPRAS, 2006.

2. Potter M, Irvin L, Grobbelaar A, Dye J. Development of a proangiogenic matrix for synthetic dermis using cultured endothelial cells. Institute Pasteur, Paris, 2005. Presented by DJ

3. Potter M, Linge C, Cussons P, Dye J, Sanders R. Improving the take of dermal skin equivalents. 8th European conference of Scientists and Plastic Surgeons. Munich, October 2004.

4. Potter M. Linge C, Sanders R, Dye JF. Fibrin is a potent stimulus of endothelial migration. The 23rd European conference on microcirculation. Lisbon, September 2004.

5. Potter M, Dye JF, Linge C, Sanders R. Is collagen the optimum constituent for Dermal Skin Equivalents? The 2nd World Union of Wound Healing Societies Meeting, Paris, 2004.

6. Potter M, Linge C, Dye JF, Cussons P, Sanders R. Are we making the most of the angiogenic potential within dermal matrices? 7th European conference of Scientists and Plastic Surgeons. Geneva 2003.