AN INVESTIGATION INTO EQUINE WOUND HEALING AND SARCOID FORMATION

by

Christine Ann Cochrane

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

July 1996

ABSTRACT

Wound healing in the equine species is often complicated by the formation of exuberant granulation tissue which prevents re-epithelisation from taking place.

Another complication in equine wound healing is the occurrence of sarcoids, which are locally invasive tumours of the skin that often appear at the site of wounds. They usually appear in clusters and are most frequently seen as complications of wound healing on the head, limbs and ventral abdomen of the horse. These problems are important factors in the welfare of horses and very little is known about their causes which are poorly documented.

The aim of this study was to investigate the tissue architecture of normal skin, granulation tissue and sarcoid tissue. The localisation of extracellular matrix molecules, growth factors, and cell adhesion molecules was studied by immunohistochemistry. Pericapillary cuffs were located around blood vessels in the wound area and at the margin. These cuffs were found to be composed of fibrin, laminin, fibronectin, collagen and endothelial cell adhesion molecules. There was limited fibronectin present but an abundance of type I collagen in the wound area. Tissue in the immediate wound area also had a high growth factor profile.

Collagen, protein and TGFβ were measured in skin homogenates. Levels of hydroxyproline and TGFβ were elevated in the granulation tissue homogenates when compared to the controls. Sarcoid tissue homogenates also showed elevated levels of these molecules but to a much lesser degree than granulation tissue.

Primary cell cultures were established from normal skin, granulation tissue and sarcoids. All three cell types were dependant on serum for proliferation and differed from each other in morphology, proliferation, quantity and type of collagen produced, protein synthesis, metalloproteinase profile and the response to growth factors. The effect of exogenous growth factors on the cell cultures was investigated by measuring cell proliferation, collagen and protein synthesis. Cell morphology was altered by the addition of exogenous growth factors and the proliferation rate of the granulation tissue derived cells was inhibited. Release of type I collagen and TGFβ into the medium was also measured and found to be elevated in the granulation-tissue derived cells.

Metalloproteinases are thought to play an important role in tissue breakdown during wound healing. The nature of these metalloproteinases was investigated in serum from wound and sarcoid cases, wound fluids and cell supernatants by zymography. Two major gelatinases were detected (MMP2 and MMP9) that are known to be involved in tissue breakdown. The activity of MMP2 and MMP9 in wound fluids significantly increased in the first few days post wounding but there was a further increase later in the healing process. The synthesis and release of stromelysin showed the opposite effect.

In conclusion, the data obtained indicate that the inflammatory response in the non-healing equine wound is not being switched off. The wound actually remains inflamed indefinitely, suggesting a chronic inflammatory response. Evidence for this hypothesis has been demonstrated by the detection of high levels of gelatinases in wound fluids within a few days after wounding which decrease briefly and then further increase over time. Also, elevated levels of TGFβ in the cell culture supernatants, tissue homogenates and wound fluids of the granulation tissue suggests that there are large numbers of inflammatory cells present within the tissue itself.

CONTENTS

CHAPTER ONE
INTRODUCTION

CHAPTER TWO
METHODS

CHAPTER THREE

THE HISTOLOGY, EXTRACELLULAR MATRIX AND GROWTH FACTOR DISTRIBUTION IN EQUINE CHRONIC WOUND HEALING

CHAPTER FOUR

THE EXAMINATION OF EXTRACELLULAR MATRIX MOLECULES IN SARCOID, GRANULATION TISSUE AND NORMAL SKIN BIOPSIES BY COMPARATIVE BIOCHEMISTRY

CHAPTER FIVE

EQUINE DERMAL AND EPITHELIAL CELL CULTURE

CHAPTER SIX

THE EFFECT OF GROWTH FACTORS ON CELL PROLIFERATION AND MORPHOLOGY IN GRANULATION TISSUE, SARCOID-DERIVED AND NORMAL DERMAL FIBROBLASTS

CHAPTER SEVEN

THE SYNTHESIS OF EXTRACELLULAR MATRIX MOLECULES BY GRANULATION TISSUE AND SARCOID-DERIVED FIBROBLASTS COMPARED TO NORMAL EQUINE DERMAL FIBROBLASTS

CHAPTER EIGHT

THE ROLE OF METALLOPROTEINASES IN EQUINE WOUND HEALING AND SARCOID FORMATION

CHAPTER NINE

GENERAL DISCUSSION

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

ABBREVIATIONS

The following abbreviations have been used in this thesis:

L	Litre
ml	millilitre
µl	microlitre
g	gramme
µg	microgramme
M	Molar
mM	millimolar
µM	micromolar
min	minute
BSA	Bovine Serum Albumin
CP	Collagenous protein
DMEM	Dubecco's modified Eagle's medium
dpm	Disintegrations per minute
EACA	6-aminohexanoic acid
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
E-Selectin	Endothelial leucocyte adhesion molecule -1
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
GT	Granulation tissue-derived fibroblast

GuHCl	Guanidium hydrochloride
H & E	Haematoxylin and eosin
Hcl	Hydrochloric acid
ICAM-1	Intercellular adhesion molecule-1
kD	Kilodaltons
LSB	Laemmli sample buffer
Mwt	Molecular weight
NaOH	Sodium hydroxide
NEM	N-ethylmaleimide
NF	Normal fibroblast
NCP	Non-collagenous protein
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor
PECAM	Platelet endothelial cell adhesion molecule
PMSF	Phenylmethanesulphonyl fluoride
SDS	Sodium-dodecyl-sulphate
SDS-PAGE	Sodium-dodecyl-sulphate polyacrylaminde gel electrophoresis
SEM	Standard error of the mean
SF	Sarcoid derived fibroblast
TCA	Tetrachloroacetic acid
TEMED	N,N,N',N'-tetramethyl-ethylene-diamine
TGFβ	Transforming growth factor beta
VCAM-1	Vascular cell adhesion molecule-1

ACKNOWLEDGEMENTS

The research described in this thesis would not have possible without the patience and guidance of a number of people:

Dr Derek C. Knottenbelt for his advice, encouragement and moral support over the past three years. Everyone connected to the Department of Veterinary Clinical Sciences who have given me their help and friendship. Thanks are also extended to Professor Anderson, for allowing me to use the facilities of the Department of Veterinary Clinical Science.

Grateful thanks to Professor Malcolm J. Jackson and Dr. Stuart Carter for their advice on writing this thesis and data presentation.

My everlasting thanks Paul, Paul and Mark for their support, love and encouragement during my post graduate studies.

Finally, I would like to thank the Home of Rest for Horses for without their financial support this work would not have been possible.

CHAPTER ONE - Review of the literature

1.1 SUMMARY OF THE WOUND HEALING PROBLEMS IN THE HORSE

Equine wound healing is often more complicated than in other species. These complications often involve a condition known as 'proud flesh'in which exuberant granulation tissue formation is present. In these wounds, the granulation tissue hyper-proliferates to an excessive level thereby preventing re-epithelisation from taking place. The second type of non-healing or chronic wound commonly seen by veterinary surgeons is the indolent wound. In this case, the wound produces very little granulation tissue, it is inflamed and often infected.

Sarcoids are locally invasive tumours of the skin that often appear at the site of wounds and cause added complications. These problems account for a significant number of horses being euthanised each year. Therefore, scientific investigation into these problems was considered to be important in the general welfare of horses. As there have been very few scientific investigations conducted into equine wound healing, the following section (1.2 Principles of wound healing) refers mainly to man unless otherwise stated.

1.2 PRINCIPLES OF WOUND HEALING

There are three accepted phases in the healing of human skin wounds: (1) an inflammatory phase, (2) a proliferative repair phase and (3) a remodelling phase. Within hours of injury, surface wounds begin the repair process but the entire process may take many months.

1.2.1 Inflammatory phase

All tissue injury is associated with inflammation. Acute inflammation is characterised by a vascular and cellular response designed to protect the body against excessive blood loss and invasion by foreign substances. It also serves to dispose of dead and dying tissue in preparation for the repair process. The inflammatory phase can be divided up into vascular and cellular reactions that can last up to six hours after wounding (Clark, 1993). The duration of this phase depends on the degree of trauma, nature of injury, whether foreign substances remain in the wound site and whether infection develops. Intense vasoconstriction, platelet aggregation and activation of the clotting cascade prevent blood loss during the first few minutes after injury (Clark, 1993). Platelet aggregation and adhesion are stimulated by a number of components generated or expressed at the wound site, including thrombin and exposed fibrillar collagens (Postlethwaite & Kang, 1976; Bar-Shavit *et al.*, 1983). Platelets release their α-granules containing fibrinogen, fibronectin, thrombospondin and von Willebrand factor VIII (Lanir *et al.*, 1988) During blood clot formation fibrinogen is converted to fibrin by thrombin, which is then stabilised with covalent crosslinks by coagulation factor XIIIa transglutaminase (Ross *et al.*, 1974; Rutherford & Ross, 1976). Fibronectin is incorporated by binding to fibrin and by formation of specific crosslinks via factor XIIIa (Mosher, 1975). In the guinea pig model both fibrin and fibronectin act as a provisional matrix for the influx of neutrophils, monocytes and fibroblasts (Clark *et al.*, 1982a).

Substantial quantities of growth factors are released by platelets (Sporn & Roberts, 1986). Platelets manufacture various growth factors that are chemotactic and mitogenic for cell lines crucial to wound healing including transforming growth factors

alpha (TGFα), (Derynck, 1988; Schultz *et al.*,1991) and beta (TGFβ), (Sporn & Roberts, 1990). Red blood cells and platelets populate the wound but these cells quickly die in the hostile environment and release haemoglobin. Factors released by blood coagulation pathways and cell activation or death at the wound site stimulate the influx of inflammatory leucocytes and increase the permeability of undamaged vessels adjacent to the injured area, increasing the leakage of plasma proteins (Clark, 1993). Activated endothelia express specific endothelial cell-surface membrane receptors which recognise receptors on circulating leucocytes. These adhesion molecules are responsible for leucocyte diapedesis through the blood vessels into the wound site (Pober & Cotran, 1991). During the following twenty four hours, neutrophils and lymphocytes remove bacteria, debris and damaged tissue (Simpson & Ross, 1972). These polymorphonuclear cells are attracted into the wound area by chemoattractants produced by the complement cascade (Deuel *et al.*, 1982). Neutrophils primarily function to destroy bacteria contaminating the wound site through phagocytosis and subsequent enzymatic and oxygen free radical mechanisms but in doing so they may increase tissue damage (Simpson & Ross, 1972). A variety of proteases are expressed and released by neutrophils during the inflammatory phase, which cause extensive destruction and remodelling of the surrounding connective tissue (Hibbs *et al.*, 1985). Neutrophils stop invading the wound space if no contamination has occurred and redundant neutrophils are phagocytosed by tissue macrophages (Newman *et al.*, 1982). Monocytes continue to infiltrate the wound site irrespective of contamination, stimulated by selective chemoattractants such as fragments of fibrin, fibronectin, collagen and elastin as well as enzymatically active thrombin, complement and platelet factors (Postlethwaite,

Snyderman & Kang, 1976; Senior, Griffin & Mecham, 1980; Norris *et al.*, 1982). When activated, monocytes exhibit the same features as macrophages.

Macrophages engulf and destroy bacteria and cellular debris and produce several factors including proinflammatory mediators, chemoattractants, growth factors (Sporn & Roberts 1986) and enzymes, including matrix degrading proteases (Werb & Gordon 1975a &1975b; Wahl & Wahl, 1985). As well as facilitating the recruitment of additional inflammatory cells, these substances aid in tissue decontamination and debridement and initiate and prolong subsequent granulation tissue formation. As macrophages ingest microorganisms, they excrete products of digestion, such as ascorbic acid, hydrogen peroxide and lactic acid. Hydrogen peroxide aids in controlling anaerobic microbial growth and the build up of ascorbic and lactic acid stimulates the influx of more macrophages, thereby prolonging the inflammatory response (Niinikoski, Gottrup & Hunt, 1991). Macrophages release growth factors that stimulate fibroblasts (PDGF, TGFα, TGFβ, FGF, macrophage derived growth factors and interleukin-),(McGrath 1990). In addition to stimulating fibroblasts, these molecules act on epithelial and vascular endothelial cells, encouraging epithelisation and angiogenesis (McGrath, 1990; Ross *et al.*, 1986).

In response to the release of cytokines at the sites of inflammation, a series of cell adhesion molecules are expressed on the surface of endothelial cells and other cell types. Cell adhesion molecules are cell-surface proteins, glycoproteins or lectins that mediate cell-cell or cell-matrix binding. They play a crucial role in both acute and chronic inflammation, wound healing, and tumour metastasis. There are five families of cell adhesion molecules expressed by endothelial cells during inflammation (Swerlick

& Lawley, 1993):

a) the immunoglobulin gene superfamily

b) the selectin family

c) the integrin family

d) the cadherin family

e) the cartilage-link-protein family.

Circulating leucocytes express receptors for these endothelial adhesion molecules and their expression results in increased adhesion of leucocytes to the endothelium. With the conversion of fibrinogen to fibrin and the production of water insoluble glycoprotein (fibronectin), a fibrocellular clot forms that fills and binds the wound together (Ali & Hynes, 1978; Grinnell *et al.*, 1980) providing early tensile strength (Figure 1.1). When the clot dehydrates it forms a scab which protects the wound from external contamination but scab formation is not always necessary for effective wound healing, often wounds heal more rapidly when kept moist under a bandage (Dyson *et al.*, 1988). Macrophages have been shown to enhance scar formation in rabbit corneas (Clark *et al.*, 1976). The plugging of the damaged lymphatics which prevents fluid drainage, results in localisation of the inflammatory response characterised by swelling, redness and heat and in some cases pain resulting from pressure and chemical stimulation.

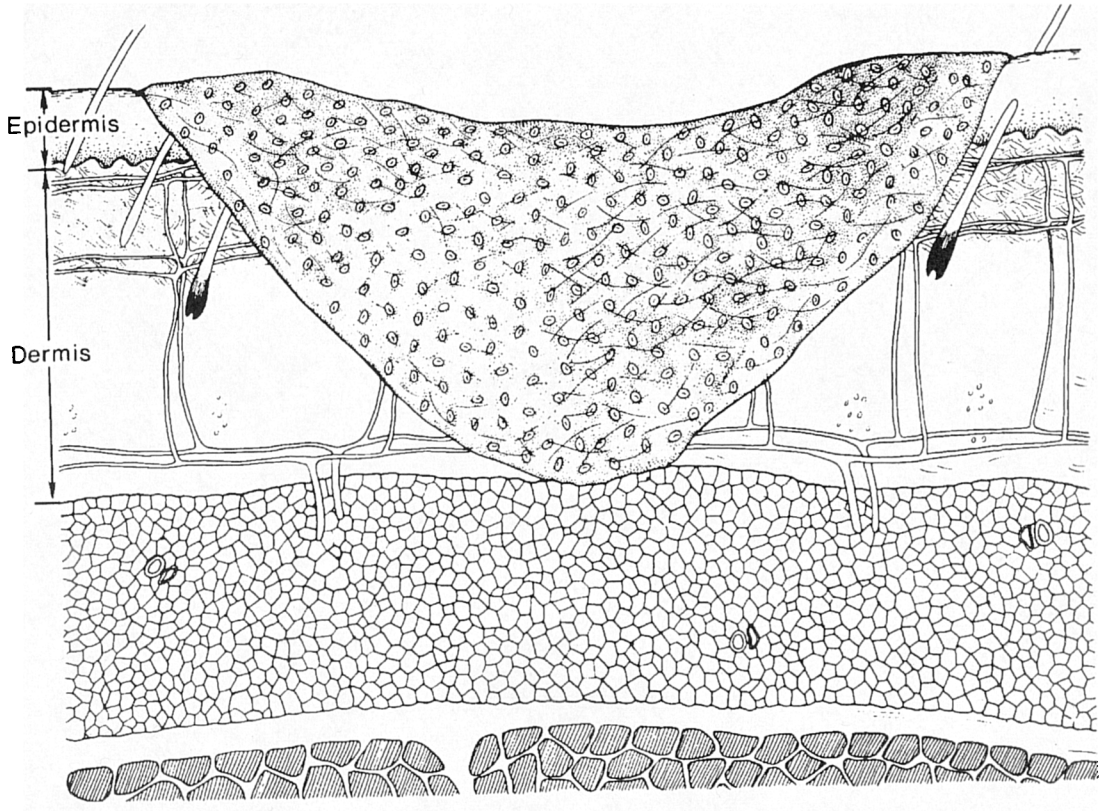
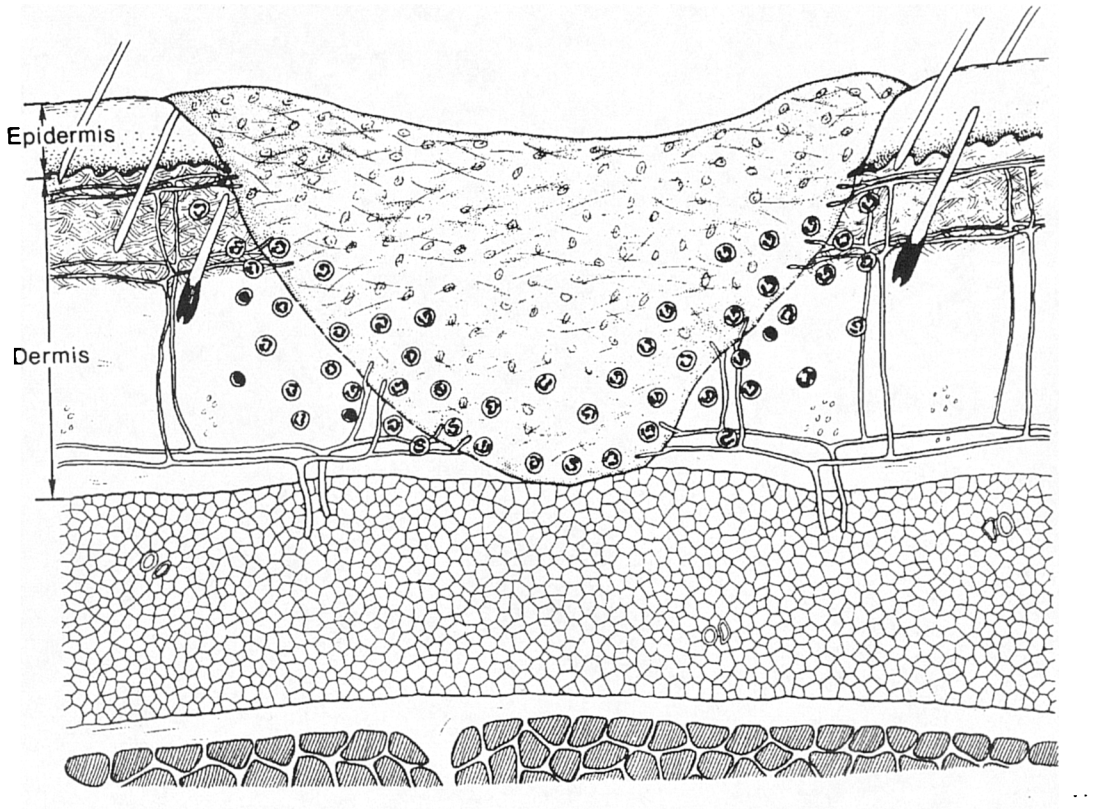


Figure 1.1

The fibrocellular clot that binds the wound together. (From Stashak TS: In: The Practice of Large Animal Surgery, Jennings PB (ed), WB Saunders. Philadelphia1984).

Figure 1.2

White blood cells migrate into the wound to begin the clean-up process. (From Stashak TS: In: The Practice of Large Animal Surgery, Jennings PB (ed), WB Saunders Philadelphia, 1984)



1.2.2 Proliferative repair phase

In man, the debridement phase begins about six hours after wounding and lasts up to twelve hours. During this phase neutrophils and monocytes that were chemotactically stimulated to migrate into the wound begin the "cleaning-up" process (Figure 1.2). The primary function of these cells is to ingest microorganisms by phagocytosis (Carrico *et al.*, 1984). An important function of the monocyte is to attract fibroblasts into the wound (Doherty *et al.*, 1987). As fibroblasts and other cells migrate into the site of injury, they begin to proliferate and the cellularity of the wound increases (Clark, 1993). This begins the proliferative and repair phase which usually begins within the first twelve hours after wounding and often lasts for several weeks. The number of inflammatory cells in the wound begins to decrease and other cells such as, fibroblasts, endothelial cells and keratinocytes continue to synthesise growth factors (Sporn & Roberts, 1986). These factors continue to stimulate proliferation, the synthesis of extracellular matrix proteins and new capillary formation. The wound is essentially watertight by 48 to 72 hours (Simon, 1988; Trott, 1991).

The early inflammatory response in normal human wounds is rapidly followed by a fibroblastic phase with the formation of granulation tissue. Granulation tissue begins to appear in the wound about three to six days after injury. The granular appearance of this tissue results from proliferating capillaries that form vascular loops (Clark, 1993). Macrophages, fibroblasts and blood vessels move into the wound space (Hunt, 1980). These loops grow behind the fibroblasts and from the intercommunication of the terminal branches of multiple blood vessels (anastomoses). The fibrin network is lysed by plasminogen activators secreted by vascular endothelial cells. The capillaries

and the lymphatics develop at a slower rate. Granulation tissue formation has several benefits, it provides a surface for epithelial cells to migrate over (Lark *et al.*,1985; Hsieh & Chen, 1983) it is resistant to infection, wound contraction is centred around its development (Gabbiani *et al.*, 1972; Majno *et al.*, 1971) and it carries fibroblasts responsible for collagen synthesis (Kurkinsen *et al.*, 1980). The horse is capable of collagen formation as early as the first day after wounding but generally in man collagen forms on the third or fourth day. Chvapil *et al* (1979) identified and increased formation of collagen and collagenase in horses' wounds, concluding that healing in the horse is an excessive and abnormal repair. Significant number of fibroblasts originating from undifferentiated mesenchymal cells in nearby connective tissue move into the avascular wound space (fibroplasia) by a combined process of migration and proliferation *in situ* (Ross & Benditt, 1961) moving along the previously formed fibrin lattice within the clot. Evidence has emerged that the wound event selects various fibroblast subtypes causing expression of certain fibroblast phenotypes from an otherwise heterogenous dermal population (Regan *et al.*, 1991). The fibroblasts main function is the synthesis of structural proteins, especially collagen, reorganisation of the provisional extracellular matrix and wound contraction, resulting in the formation of scar tissue.

Fibroblasts migrate into the wound space by retracting their endoplasmic reticula and Golgi apparatus to perinuclear locations and forming large actin bundles throughout the peripheral cytoplasm which orientate parallel to the long axis of the cell (Gabbiani *et al.*, 1978). These phenotypically altered fibroblasts are known as myofibroblasts (Gabbiani *et al.*, 1978) and are both motile and contractile. Cell movement is terminated by contact inhibition of like cells. Immediately after entering the wound the fibroblasts

begin secreting a ground substance, containing protein polysaccharide and glycoprotein, which is necessary for collagen deposition (Welch *et al.*,1990). The formation of collagen begins about the fourth or fifth day and is initiated by the extrusion of the tropocollagen molecule into the extracellular spaces. As these immature fibrils are produced they begin to bind together to form a mature collagen fibre by increasing the collagen content and decreasing the ground substance (Birk *et al.*,1989; Yamaguchi *et al.*, 1990).

Wound contraction begins between the third and fourth day and appears to be independent of the epithelisation that is occurring at the same time. Skin movement results from the contractile properties of myofibroblasts that are found in granulation tissue adjacent to the wound (Gabbiani, 1979). They have good cell-cell and cell-stroma contact and have many properties of smooth muscle including the ability to contract (Welch *et al.*, 1990). As these cells contract they draw the surrounding full-thickness skin towards the centre of the wound thereby temporarily, stretching and thinning the surrounding skin. New collagen is added to the dermis and new cells are added to the epithelium. Skin thickness is restored and the skin tension is reduced. The shape of the wound defect may have an effect on its ability to contract. Wound contraction ceases when contact inhibition of cells occur, tension of surrounding skin equilibrates with pulling forces of contraction (Singer *et al.*,1984; Welch *et al.*, 1990). Exuberant granulation tissue impedes the wound's ability to contract or when full-thickness skin grafts are applied to the wound before the fifth day of healing. The first visible sign of repair, epithelisation, is recognised about twelve hours post wounding by a flattening of the rete pegs of the epidermis. This flattening forces adjacent cells towards the wound

edges. Simultaneously, basal cells of the epidermis begin to separate, duplicate and migrate towards areas of cell deficit (Figure 1.3). Epithelial cell migration appears to be stimulated by contact with other similar cells, loss of neighbouring cells or by a number of chemoattractants although some may migrate to the centre of the wound independently. If the basement membrane remains intact then hemidesmosomes that link epithelial cells with the basement membrane are temporarily disrupted during cell migration. If however, the basement membrane is destroyed, by injury or enzymatic degradation, epithelial cells migrate over a provisional matrix of fibrin, fibronectin and tenascin (Clark *et al.*, 1982a; Mackie *et al.,* 1988).

Basement elements including collagen type IV, heparan sulphate proteoglycan and laminin must be replaced. The epidermis begins a regenerative phase, in which fully differentiated stratum corneum and specific markers, such as mature isoforms of keratin and involucrin become re-established (Davidson, 1992). Impaired re-epithelisation characterises many chronic wounds, where resurfacing may fail to occur and the overall appearance of the wound bed remains unchanged for months. Under these circumstances, all epidermal regrowth must occur from the wound margin along newly formed granulation tissue and basement membrane components (Davidson, 1992). Figure 1.3 demonstrates the sequence of epithelisation in partial-thickness and full-thickess wounds. In culture, keratinocytes fail to migrate on a substrate of laminin, but do so readily when in contact with fibronectin, and dermal interstitial and basement membrane-specific collagen type IV (Sarret *et al.*, 1992a).

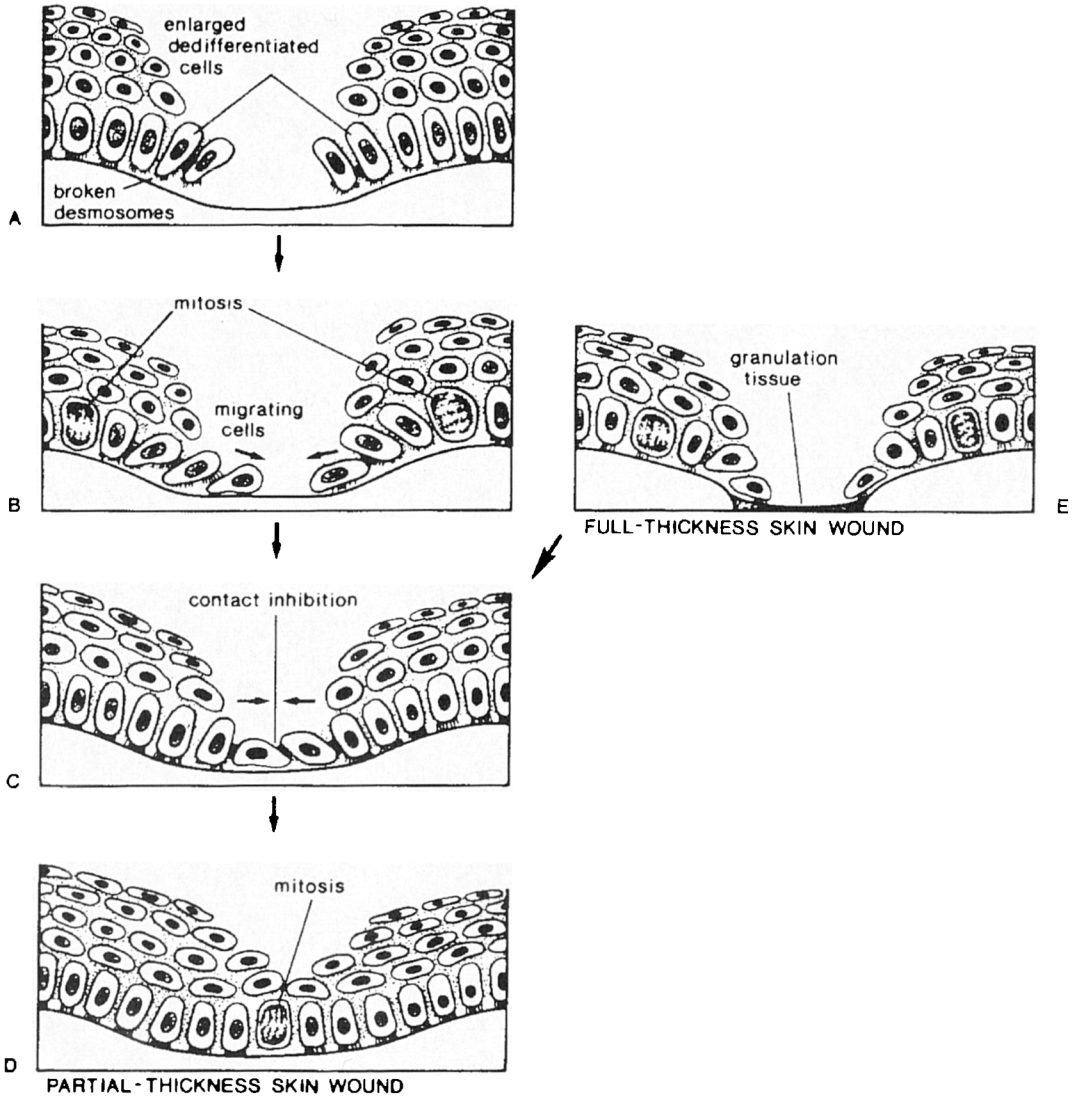


Figure 1.3

The sequence of epithelialisation in a partial-thickness wound A-D. A full thickness open wound (involving the dermis) E. (After Swain SF: Surgery of Traumatised skin, WB Saunders, Philadelphia, 1980).

If a scab is present, the epithelial cells must migrate underneath the scab and detach it by secreting proteolytic enzymes. Once epithelialisation is complete the scab falls off. Epithelialisation may be arrested prematurely by infection, excessive production of granulation tissue, repeated dressing changes (causing trauma to the wound), extreme hypothermia, desiccation of the wound surface and reduction in oxygen tension. In a full thickness open wound a granulating bed must be formed before epithelisation can take place (Figure 1.3). There is usually a lag phase of four to five days before the epithelial cells begin to migrate. It may take weeks to months for large defects to be completely covered. When several layers of epithelial cells cover the wound, cell differentiation and keratinization begin (Figure 1.4). Eventually, these cells are laid down on a smooth surface of connective tissue, which forms a loose bond, so that the epithelial cells are striped off easily. The surface lacks the structure of normal skin (Figure 1.5) and is referred to as scar tissue (Figure 1.6).

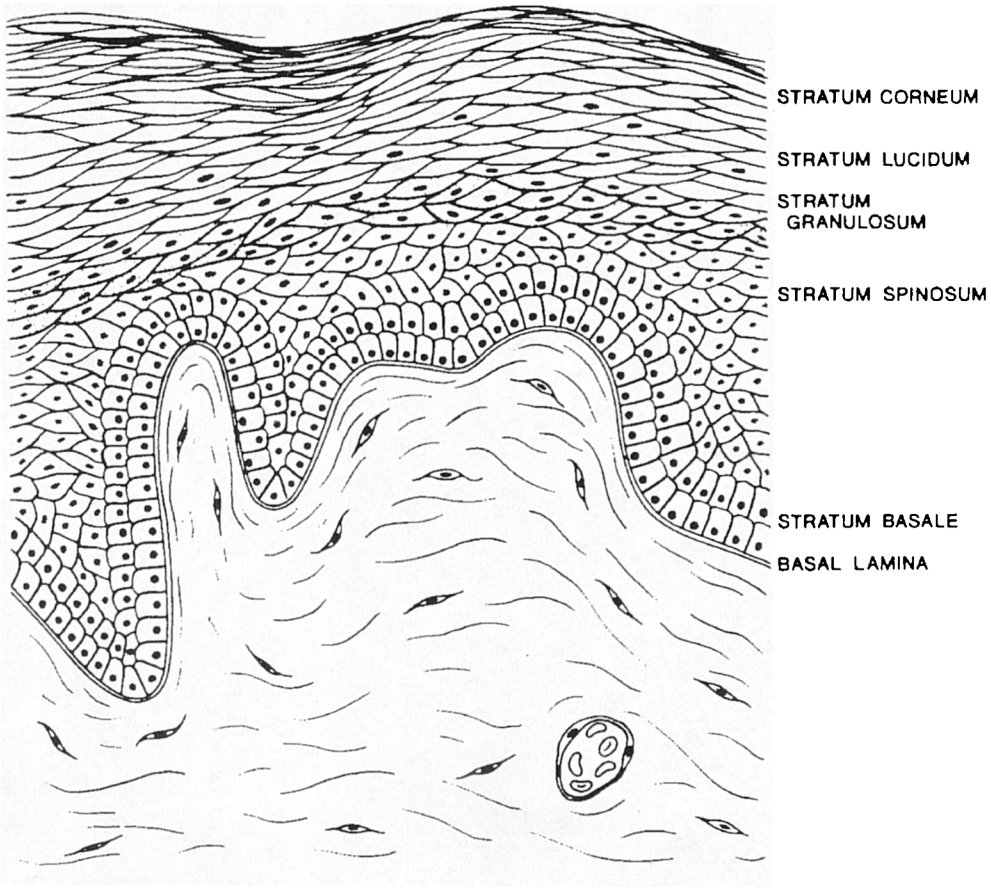


Figure 1.4

The layers of the epidermis. The stratum spinosum and stratum basale collectively are referred to as the stratum germinativum. After wounding, the cells begin to separate, migrate and duplicate. The rete pegs begin to flatten out, forcing cells toward the region of cell deficit. (From Stashak TS: In: The Practice of Large Animal Surgery, Jennings PB (ed), WB Saunders Philadelphia, 1984).

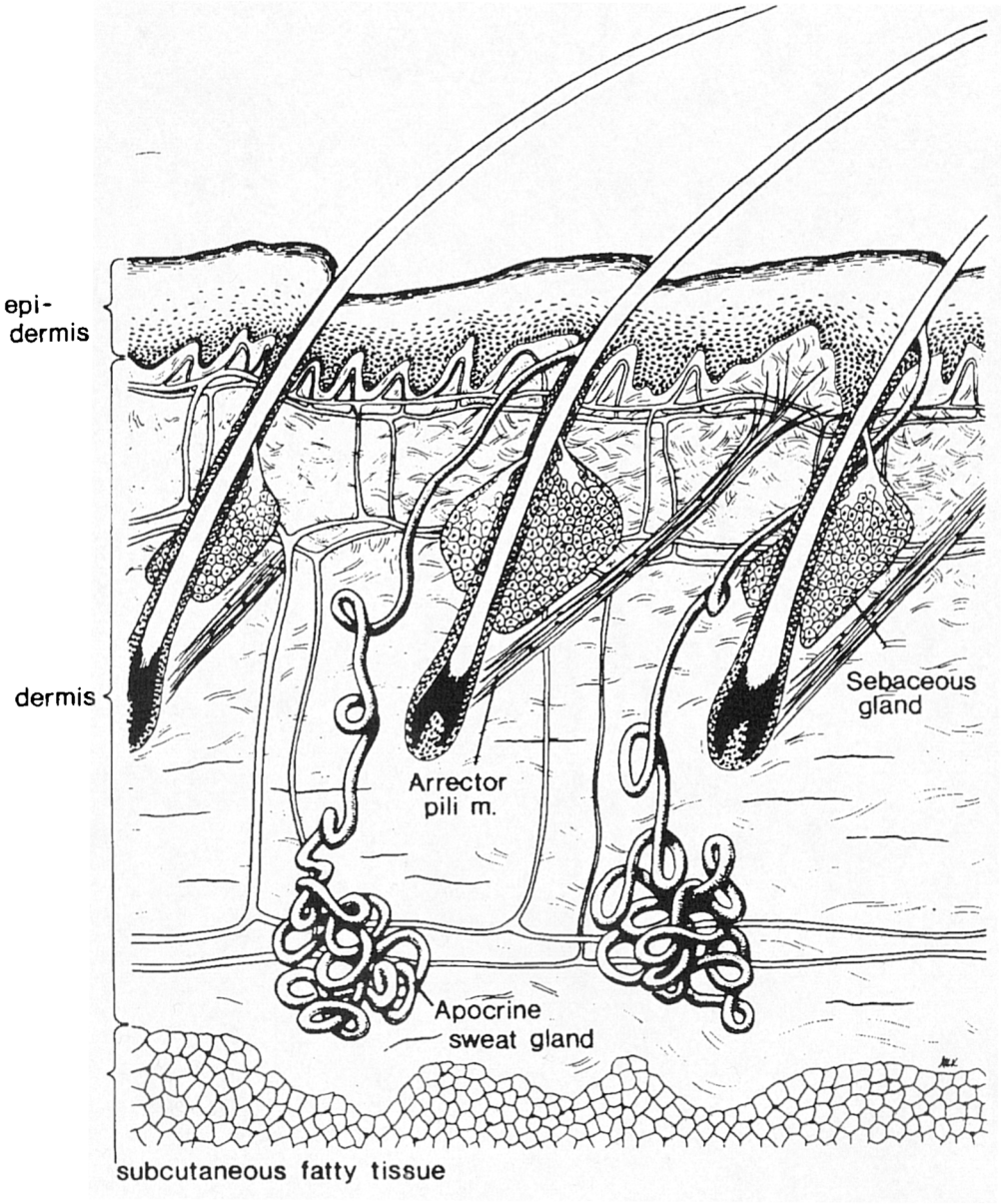


Figure 1.5

The layers of skin, the adnexa in the dermis and the subcutaneous tissue. (From Stashak TS: In: The Practice of Large Animal Surgery, Jennings PB (ed), WB Saunders Philadelphia, 1984).

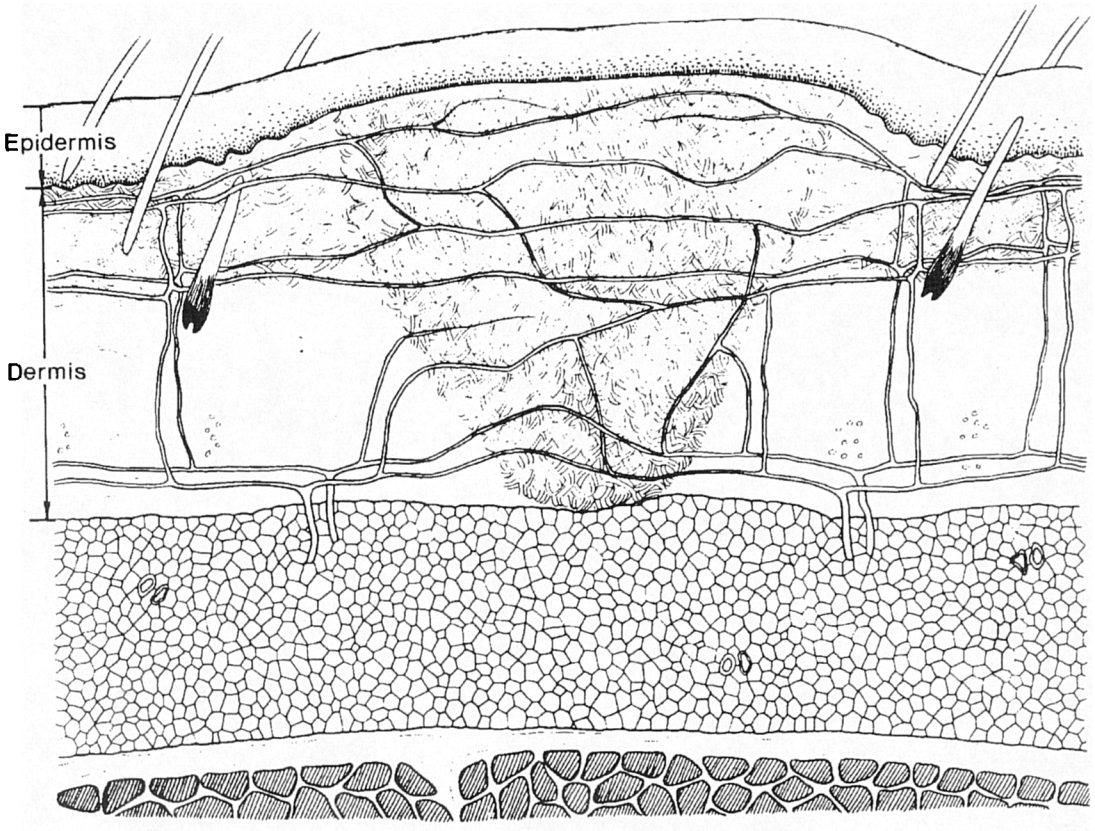


Figure 1.6

Healing of a full-thickness open wound left to heal by second intension.The dermis heals with scar tissue formation and lacks adnexa. The wound has epithelialised and is called scar tissue epithelium. (From Stashak TS: In; The practice of Large Animal Surgery, Jennings PB (ed),WB Saunders Philadelphia, 1984).

1.2.3 Remodelling phase

After the initial scar forms, proliferation and neovascularisation cease and the wound enters the remodelling phase, which usually lasts several months (Compton *et al.*, 1989). The remodelling or maturation phase is characterised by a reduction in fibroblast numbers and a balance between synthesis of new components of the scar matrix and their degradation by proteases such as collagenase (Compton *et al.*, 1989) The matrix is constantly altered with relatively rapid elimination of most fibronectin and hyaluronan, slow accumulation of large fibrous bundles of type I collagen with relatively little collagen type III (Clark, 1993). Increased tensile strength is achieved by the presence of these molecules along with proteoglycan deposition. Collagen synthesis and lysis are in balance in normal wound healing also, the granulation tissue is completely replaced by a densely woven meshwork of collagen fibre bundles with reduced vascularisation that is characteristic of scar tissue. Functionally orientated collagen fibres begin to predominate as nonfunctional ones are dissolved (Hasty *et al.*, 1986). The tensile strength of rat wounds increases despite the reduction of fibroblasts, blood vessels and collagen fibres (Levenson *et al.*, 1965). This increase in tensile strength results from the alignment of collagen along lines of tension, intramolecular cross linking assisted by lysyl oxidase and the formation of more bundles (Bailey *et al.*, 1975). The spacing between the collagen bundles becomes less distinct as the fibres interlock. The scar formation increases in tensile strength over a prolonged period of time but it will remain 15 to 20 % weaker than the surrounding tissue (Levenson *et al.*, 1965). The final component to be deposited in wounds is elastin (Davidson, 1992).

1.2.4 The role of fibronectin in wound healing

Fibronectin is a multi functional glycoprotein in which functional complexity is parallelled by structural diversity. Multiple forms of fibronectin are generated by cell-type specific alternative splicing (Mosher, 1989; Hynes, 1990). Several cell types are known to synthesise and secrete fibronectin in culture: fibroblasts (Hynes, 1973; Ruoslahti & Vaheri, 1974, Yamada & Weston, 1974), macrophages (Colvin *et al.*, 1979; Alitalo et al., 1980), endothelial cells (Jaffee & Mosher, 1978; Macarak *et al*, 1978) and keratinocytes (Kubo *et al.*, 1984). During acute wound healing, fibronectin is present in the initial plasma clot, on the surface of platelets, attached to collagen, deposited beneath the migrating epidermis and associated with monocytes, fibroblasts and capillary ingrowth. The fibronectin matrix is restricted in normal human skin (Clark, 1983; Couchman *et al.*, 1990), except where fibronectin is associated with dermal fibroblasts. The fibronectin content of the wound matrix is much higher than that of adjacent tissue (Grinnell *et al.*, 1981). The factors in a wound that regulate fibronectin production are unknown. However, *in vitro* thrombin and TGFβ can stimulate fibroblasts to synthesise and secrete fibronectin (Mosher & Vaheri, 1978; Ignotz & Massague, 1986). Fibronectin has multi functional roles during wound healing, such as enhancing phagocytosis, cell adhesion and migration and matrix formation (Clark 1988). Fibronectin may facilitate debridement of a wound by its ability to promote monocyte/macrophage, fibroblast and epithelial cell phagocytosis (Brown, 1986; Clark 1988). Cell and fibronectin interaction is accomplished by specific domains of the fibronectin molecule and cell surface receptors (Yamada, 1989). Many *in vitro* studies have shown that fibronectin mediates the binding of human fibroblasts (Grinnell & Feld, 1979), keratinocytes (Clark *et al.*,

1985; O'Keefe *et al* 1985), endothelial cells (Macarak & Howard, 1983; Clark *et al.*, 1986), and monocytes/macrophages (Bevilacqua *et al.,* 1981) to various extracellular matrix substrata.

In vivo studies have shown that fibronectin appears at times and sites in wounds where cells are migrating and proliferating, around ingrowing fibroblasts, beneath the migrating epidermis and within the walls of new blood vessels (Grinnell *et al.,* 1981; Clark *et al.*, 1982a; Clark *et al.*, 1982b). Fibronectin deposition in wounds precedes collagen deposition (Kurkinsen *et al.,* 1980) and wound contraction (Welch *et al.*, 1990). Both plasma and cellular fibronectins can become incorporated into extracellular matrices (Wilson & Schwrazbauer, 1992). Fibronectin is multi functional in the wound environment and is an essential component of normal wound repair. It is therefore, possible that problems in its production or excessive degradation may lead to delayed wound healing as seen in some chronic wounds.

1.2.5 Collagen in wound repair

Collagens are a family of closely related triple-chain glycoproteins found in the extracellular matrix and involved in tissue repair. There are three types that are mainly involved in wound healing. These are types I, III, and IV. Type I collagen is the major structural component of skin, tendon, bone and many minor structures. Type III collagen is also present in skin in association with type I, although that association is not fully understood. The ratio of type I to type III varies. Normal mammalian skin contains approximately 80-90% type I collagen and 10-20% type III collagen (Epstein, 1974; Miller, 1976). Type III is usually considered to be in the thin reticular fibrils and type I in the larger fibrils (Martin *et al.,* 1985). They are similar molecules and there is some

evidence that they may coexist in single fibrils (Henkel and Glanville, 1982).

Type IV collagen, together with other components, including a unique heparin sulfate proteoglycan and the glycoprotein laminin, makes up both the epidermal and the endothelial basement membranes.

Types I and III collagen molecules are partially flexible rods about 300nm in length and 1.5nm in diameter. Type IV collagen is highly glycosylated, has a 400nm long helix that is interrupted by several short nonhelical regions and contains a globular knob at its carboxyl-terminal end. Each molecule consists of three polypeptide chains. These chains form an unbroken triple helix except for short nonhelical regions (about 6-26 amino acid residues) at both ends. The collagen polypeptide chains are synthesised as larger precursors or pro-α-chains with carboxy and amino terminal peptides (Figure 1.7). Collagen α-chains are composed of a third glycine, a third proline and a third other amino acid. Glycine occurs in every third position so making a repeating structure of gly-X-Y. Proline or lysine are usually found in the Y position. Some of the proline and lysine residues are hydroxylated through the action of specific enzymes, prolyl hydroxylase or lysyl hydroxylase in the endoplasmic reticulum before the pro-α-chains associate to form a triple-helix. Molecular or dissolved oxygen is needed for the reaction, as well as cofactors, ferrous iron, copper, zinc, alpha keto glutarate and a reducing agent such as ascorbic acid or light activated riboflavin (reviewed by Grant & Prockop, 1972).

Hydroxyproline and hydroxylysine are almost unique to collagen. There is indirect evidence that the hydroxyl groups of hydroxyproline residues form interchain hydrogen bonds that help stabilise the triple helix. Hydroxylation of the lysyl residues provides a substrate for glycosylation and for the formation of certain covalent

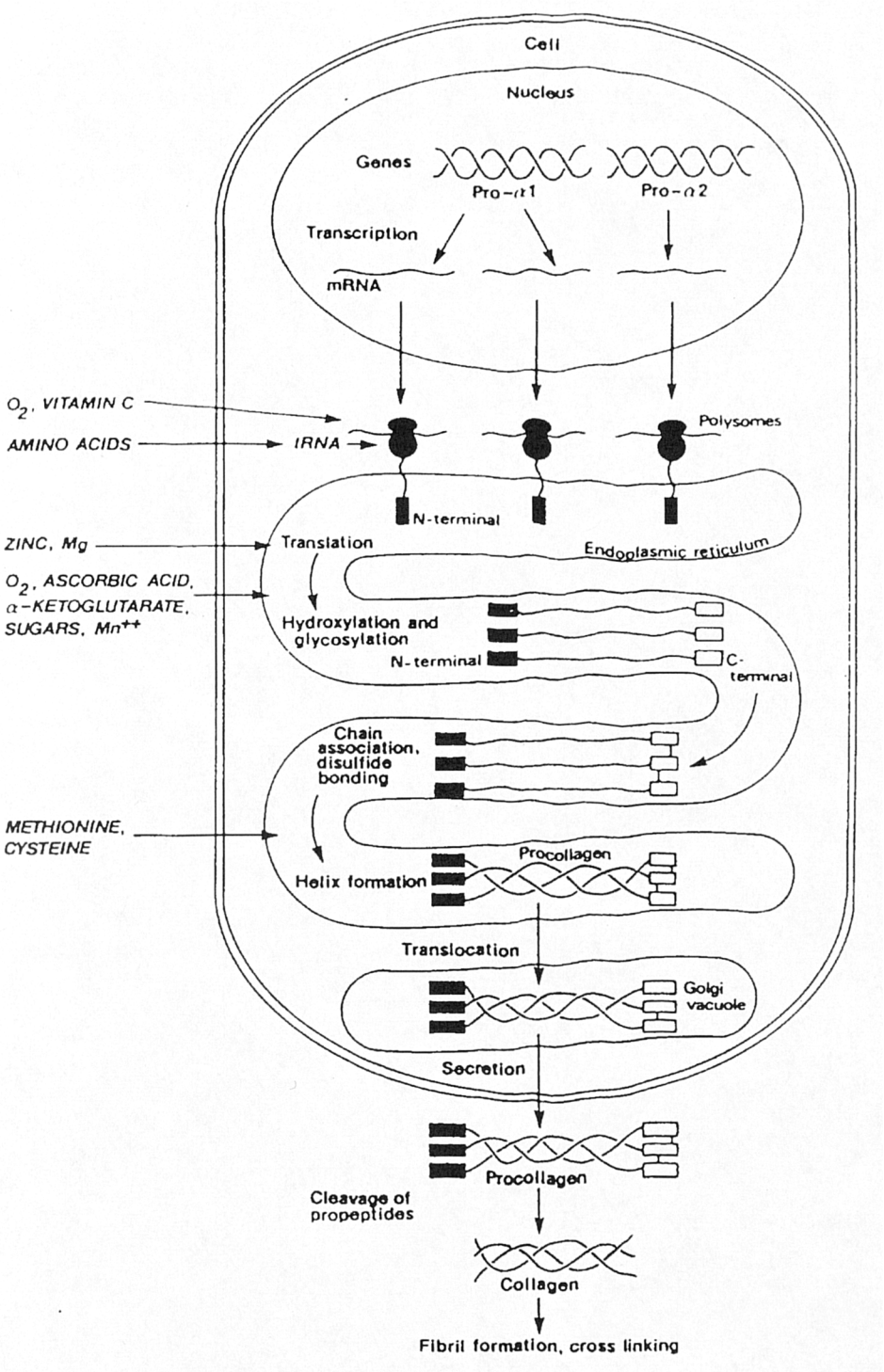


Figure 1.7

Diagrammatic representation of steps in collagen synthesis from translation to secretion (reproduced from Hunt & Van Winkle 1979).

intermolecular crosslinks, an important determinant of collagen fibril tensile strength. Each pro-α-chain combines with two others through the carboxy-terminal propeptides to form a trimeric disulphide bond. The trimer rapidly folds into a structure that is stabilised by interchain hydrogen bonded triple-stranded, helical molecule or procollagen. Procollagen is glycosylated in the Golgi apparatus before being secreted by exocytosis into the extracellular space.

During secretion, the terminal peptides of procollagen are removed by a specific proteolytic enzyme or procollagen peptidase. The resulting molecule is tropocollagen. Strong intramolecular cross-links form within the single tropocollagen molecules as tropocollagens spontaneously aggregate with one another in an overlapping array to form fibrils. After collagen fibres are formed in the interstitial space, they are strengthened by the formation of covalent cross-links within and between the constituent collagen molecules.

The function of collagen in wound repair falls into four categories:

1) inflammation

2) neomatrix formation (synthesis, deposition and remodeling)

3) wound contraction

4) re-epithelisation

These processes overlap during the course of repair, pre-existing or newly synthesised collagen plays an important role in each of them (Peacock, 1973; Carrico *et al.*, 1984).

A) Role of collagen in inflammation

Fibrillar collagens (types I and III) play a role in the initial stages of wound healing since they are believed to be a key element involved in the promotion of platelet

aggregation following vascular injury (Shoshan, 1981). The binding of platelets to fibrillar collagen in the surrounding connective tissue results in the release of several large glycoproteins such as fibronectin and thrombospondin, the latter has been implicated in the subsequent aggregation of platelets to one another (Jaffe *et al.,* 1982). Platelet aggregation results in the release of factors, which are chemotactic for inflammatory cells, as well as PDGF and fibronectin, which have been shown to be chemotatctic for fibroblasts and smooth muscle cells (Gauss-Muller *et al.*, 1980; Grotendorst *et al.*, 1981; 1982; Seppa *et al.,* 1982).

B) Neomatrix formation

The migration of fibroblasts into the wounded area and rapid vascularisation signal the synthesis and deposition of collagenous and non-collagenous extracellular matrix proteins in the wound bed. This process of fibroblast invasion and the deposition of a collagen-based extracellular matrix has been termed fibroplasia. If neovascularisation occurs also, the process is called granulation tissue formation. This phase usually begins 3-5 days after wounding and may persist for 10-12 days, during which time there is rapid synthesis of type I and III collagen and an associated increase in the tensile strength of the wound. Remodelling and cross-linking of the collagen follows fibroplasia and may continue for up to 2 years. It results in the generation of fibrillar collagen bundles or fibres. This remodelling phase ultimately results in the formation of a fibrous connective tissue commonly known as scar tissue.

C) Contraction

The role of type I and III collagen in the contraction process of wound healing is not fully understood. It is thought that the surrounding extracellular matrix, not involved

in the wound healing process and connective tissue deposited in the wound bed play an important role in the contractile process. These matrices provide the anchoring points and connecting cables to which contractile cells (myofibroblasts) bind and attempt to reduce the wound volume through an active contraction process (Gabbiani *et al.*, 1972). Several investigators have shown that contraction can be modelled *in vitro* by placing fibroblasts on or in collagen gels (Steinberg *et al.,* 1980; Grinnell and Lamke, 1984). Wound contraction is a early event in the wound healing process which involves an interaction between myofibroblasts and the extracellular matrix. Scar contracture however, is a later event associated with scar remodelling.

D) Re-epithelialisation

The type I and III collagenous matrix of early granulation tissue synthesised by fibroblasts in the wound, in conjunction with non-collagenous proteins such as fibronectin and fibrin, provides support for epidermal cell migration and proliferation (hyperplasia) (Clark *et al.*, 1982a; Woodley *et al.*, 1985). Exposure of a granulating wound to air results in the formation of a scab (eschar) which is composed primarily of dead cells and dehydrated serum and fibrin, which attaches itself to the underlying granulation tissue. Epithelial cells burrow between the eschar and granulation tissue by expressing collagenase and other proteases dissolving the collagenous matrix as they move (Harris and Krane, 1971). Collagen V is synthesised by epidermal cells during this migratory phase (Stenn *et al.*, 1979). Once wound closure is complete the scab is sloughed off. The provisional matrix in wound closure, provided by the early granulation tissue and serum-derived components is different from the basement membrane, containing type IV collagen. This results in different cellular behaviour characterised by

lateral cell movement onto the wound bed and cell proliferation rather than the vertical movement and terminal differentiation characteristic of epithelial cells when they rest on the intact basement membrane (Woodley *et al.*, 1985). Once cell closure of the epidermis is complete, a basement membrane is rapidly synthesised. This signals the end of fibroplasia and the beginning of remodelling and maturation of the wound.

E) Regulation of collagen production

Platelet derived growth factor (PDGF), has a potent chemotactic and mitogenic activity which is probably responsible for its ability to enhance collagen synthesis in the wound by promoting fibroblast invasion and proliferation .

Another platelet factor, TGFβ, has been reported to enhance the rate of connective tissue deposition in the rat subcutaneous model (Sporn *et al.*, 1983; Lawrence *et al.*, 1986).

These investigators showed that the addition of TGFβ largely reversed the decreased level of collagen synthesis observed in the Adriamycin treated animals. The combination of TGFβ, PDGF and epidermal growth factor (EGF) showed an even greater effect and restored collagen synthesis to 86% of that observed in control animals. *In vitro* experiments showed that the addition of very low levels of TGFβ to fibroblasts in culture can significantly increase the rate of new collagen synthesis per cell (Ignotz and Massague, 1986; Roberts *et al.* , 1986). Thc ability to demonstrate a positive effect of growth factors on the wound healing response in animals is not only dependent on the factors chosen but also on the experimental animal models used. Using full-thickness skin wounds in the Syrian hamster, Leitzel *et al* (1985) were unable to show positive

effects of a number of topically applied growth factors, including PDGF, EGF, and FGF on the wound healing response. Experiments showed that macrophages, which were cultured *in vitro*, produced and released into the media a factor that caused fibroblast proliferation *in vitro* (Liebovich and Ross, 1976). This observation has been confirmed since in several different laboratories (reviewed by Liebovich, 1984). This factor was designated macrophage-derived growth factor (MDGF) by Martin *et al.*, in 1981. Further evidence was provided that MDGF was not a single protein but rather an activity provided by PDGF-like (Shimokado *et al.*, 1985) and FGF-like (Baird *et al.*, 1985) proteins that are synthesised and secreted by macrophages. These factors promote collagen synthesis by increasing the number of connective tissue cells in the wound site. Macrophages synthesise and secrete a TGFβ-like protein (Assoian *et al.*, 1987), which can increase the rate of collagen synthesis by fibroblasts (Ignotz and Massague, 1986; Roberts *et al.*, 1986). Macrophages then, play an important role in fibroplasia and collagen deposition during the intermediate stages of wound healing by secreting a host of factors that enhance fibroblast proliferation. It is evident that macrophages promote fibroblasts to synthesise and secrete extracellular matrix proteins.

It has been hypothesized that reduced oxygen tension in the wound, due to high metabolic activity of cells in the wound bed, also plays an important role in the wound healing process (Hunt *et al.*, 1984). It is suggested that reduced oxygen tension results in local hypoxia and lactate production, which in turn promotes new collagen synthesis (Hunt *et al.*, 1978; Green & Goldberg, 1964; Langness & Udenfriend, 1973; Levene and Bates, 1976).

Collagenase, which is synthesised by granulocytes and macrophages, as well as

fibroblasts and epithelial cells, is a primary mediator of collagen turnover. The rate of collagen degradation changes as wound healing proceeds, starting out at a relatively slow rate during the early stages of the process and increasing as the wound matures (Zeitz *et al.*, 1978). This protease is secreted as a zymogen that can be activated by certain other proteases such as plasmin (Werb *et al.*, 1977). This activation of the collagenase, as well as inhibition of its enzymatic activity by protease inhibitors such as α_2-macroglobulin, have been proposed to play critical roles in the regulation of expression of collagenolytic activity *in vivo* (Diegelmann *et al.,* 1981).

F) Abnormal wound repair - collagen synthesis and deposition

Scar tissue is the end product of the wound healing events previously described. The proportions of the various components and their organisation in scar tissue differ from normal dermis. The collagenous matrix of the dermal scar is arranged into thick bundles that are parallel to the epidermis, whereas normal dermis lacks this type of organisation. The quality of the scar is related to the regulation of collagen production during wound healing and the balance between collagen synthesis and collagen degradation during the later stages of the process. The scar is of course, an imperfect substitute for the original tissue, since it has a lower breaking strength, serves as a diffusional barrier to nutrients and oxygen and often results in deformation and reduction of function of the original tissue (Shoshan, 1981; Chvapil and Koopmann, 1984).

G) Keloid and hypertrophic scars

When the equilibrium between collagen synthesis and collagen degradation is not in proper balance, collagen overproduction occurs, which results in the formation of keloid

or hypertrophic scars. Exuberant collagen production within the boundaries of the original wound is classified as a hypertrophic scar whereas excessive collagen production beyond the border of the original wound is classified as a keloid scar (Peacock *et al.*, 1970). Similarities to these keloid scars have been seen in the exuberant granulating wounds of horses as previously described. Collagen in hypertrophic and keloid scars is not less organised than in mature scars of skin, but has a different organisation with unique structures (Kischer *et al.*, 1982). These structures were described as three-dimensional cigar-shaped collagenous bundles that run parallel to the skin surface and tend to flatten as the scar matures.

It was proposed by Kischer and co-workers (1982), that hypertrophic and keloid scar development is related to interruptions of microvasculature regeneration. In situations in which microvascular occlusion occurs, low O_2 and high CO_2 levels result in promoting excessive collagen and proteoglycan synthesis. This hypothesis provides and explanation for the basis of excessive connective tissue deposition during wound healing but does not however, provide an explanation as to why this process is so highly variable in individuals. Also, in the case of the equine species it does not explain why wounds on the lower limb have a tendency to hyper granulate as opposed to other sites of the body.

An alternative explanation for the excessive connective tissue deposition seen in these scars is that there is variability in the remodelling of the extraccllular matrix as wound healing progresses.

1.2.6 Growth factor production

Growth factors mediate many if not all of the cellular functions involved in

wound healing. They are proteins generally weighing between 4000 and 60,000D and they can affect cellular activities in minute concentrations. The release of these molecules appears to be closely regulated but how this occurs has not yet been demonstrated. In man, growth factors produced by a variety of inflammatory cells including TGFβ, PDGF, and FGF accumulate in the acute wound and are thought to play a major role in tissue inflammation and repair (Wahl *et al.*, 1989). Table 1 lists some of the growth factors and their presumptive roles in wound healing processes. Cellular function can be affected through endocrine, paracrine, autocrine or intracrine mechanisms. Endocrine functioning growth factors are carried to the target cell via the bloodstream and affect cells at a distance from the factor generating cell. Factors functioning in the paracrine fashion affect cells in the same location in which they are released. Autocrine factors are released by, and affect the function of, the same cell. Some transformed fibroblasts respond to factors that are never secreted but instead function within the cell itself via an intracrine mechanism. The ability of a growth factor to affect a cell depends on whether the cell has receptors for that particular growth factor.

Table 1. Growth factors influencing wound healing

FACTOR	SOURCE	TARGET RECEPTOR
TGFβ (transforming growth factor beta)	Platelets, macrophage, lymphocyte, bone, most tissues	All cells
PDGF (platelet-derived growth factor)	Platelets, macrophage, endothelial cell, smooth muscle cell	Fibroblast, smooth muscle cell, Glial cells
aFGF and bFGF (fibroblast growth factor)	Macrophage, cartilage, brain	Endothelial cell, fibroblast, chondrocyte
EGF (epidermal growth factor)	Saliva, urine, Submandibular gland	Epithelial cell, fibroblast
IGF-1 (insulin-like growth factor)	Plasma, liver, fibroblast	Fibroblast, endothelial cell, fetal tissues

Receptor binding generates a series of signals within the cell that result in a specific response. They often function by activation of tyrosine kinase. Extracelluar matrix may affect the solubility of growth factors and therefore may influence how much of a factor is available to influence cellular activity. It may also function as a reservoir for growth factors by binding them and then releasing them in a controlled fashion. The matrix can also influence the type of response a cell gives to a specific factor (Nishiyama *et al.*, 1991).

A) Epidermal growth factor - EGF

Epidermal growth factor which is identical to urogastrone (Read, 1987), is a 53 amino acid polypeptide that was originally isolated from the submaxillary glands of mice (Cohen, 1962). It is found in a wide variety of tissues (Oka & Orth, 1983; Kasselberg *et al.*, 1985; Cohen, 1986; and Cohen, 1987) and is released by platelets when they degranulate (Pesonen *et al.*, 1991). Most cells have receptors for it, though the biological

role of EGF for many cells is not known. Epithelial cells have a large number of receptors (Nanney *et al.*, 1984). Significant numbers are also present in endothelial cells, fibroblasts, and smooth muscle cells (Lim & Hauschka, 1984). It is chemotactic for epithelial cells (Blay & Brown, 1985), endothelial cells (Grotendorst *et al.*,1989) and fibroblasts (Westermark & Blomquist, 1980). EGF is a potent mitogenic stimulant for epithelial cells, endothelial cells and fibroblasts (McAuslan *et al.*, 1985; Nakagawa *et al.*, 1985). It also stimulates angiogenesis (Schreiber *et al.*, 1986) and collagenase activity (Chua *et al.*, 1985).

B) Fibroblast growth factor - FGF

Fibroblast growth factor was originally discovered as a mitogen for mesenchymal cells and was also found to stimulate angiogensis and other wound healing functions (Gospodarowicz, 1974; Gospodarowicz *et al.*,1987). Both an acidic FGF and a basic FGF (aFGF and bFGF) have been identified. Acidic and basic FGF have 50% homology, though bFGF is an approximately 10 times more potent angiogenic stimulant. Both types have been isolated from a wide variety of cells. Endothelial cells both synthesise and respond to FGF (Vlodavsky *et al.*, 1987a). Both aFGF and bFGF stimulate endothelial cell proliferation and motility and thus contribute to wound neovascularisation (Gospodarowicz *et al.*, 1987). *In vitro*, FGF affects cell morphology, proliferation , fibroblast migration and the production of proteases (Pierce *et al.*, 1992). However, during wound healing, only keratinocyte growth factor (KGF or FGF7) mRNA expression was significantly increased and localised in the dermis at the wound edge and in the hypodermis below the wound. The mRNA encoding the receptor of this growth factor was found in the epidermis, suggesting that basal keratinocytes are stimulated by

dermally derived KGF during wound healing (Werner *et al.*, 1992). Basic FGF also stimulates collagen synthesis and wound contraction. (McGee *et al.*, 1988; Klingbeil *et al.*, 1991; Slavin *et al.*, 1992), epithelisation (O'Keefe *et al.*, 1988; Hebda *et al.*, 1990), fibronectin and proteoglycan synthesis (McGee *et al.*, 1988).

C) Platelet derived growth factor - PDGF

Platelet derived growth factor was the first factor to be identified (Ross & Vogel, 1978) and was localised in the alpha granules of platelets (Antoniades, 1981). It is a 30,000 to 32,000 D glycoprotein and consists of two disulphide linked subunits, A and B, with a 56% homology. The subunits are linked together as AA or BB homodimers or AB heterodimers (Johnsson *et al.*, 1982). The heterodimer is the primary product of human platelets (Hammacher *et al.*, 1988). Although the biologic activities of the three isoforms are basically the same (Heldin *et al.*, 1988), the A and B subunits have slightly different receptors, and the binding of the B receptor appears to be the more potent stimulant for mitogenesis (Martinet *et al.*, 1986). Tumours, endothelial cells and macrophages (Martinet *et al.*, 1986; Shimokado *et al.*, 1985), smooth muscle cells (Walker *et al.*, 1986) and platelets all secrete PDGF-like factors. During natural wound healing, PDGF (Ansel *et al.*, 1993) and PDGF receptor mRNA's were expressed in skin epithelial cells and PDGF mRNA was seen in connective tissue fibroblasts (Antoniades *et al.*, 1991). PDGF also activates TGFβ expression and may also influence cellular activity (Pierce *et al.*, 1989b).

PDGF stimulates the chemotaxis of neutrophils and macrophages (Deuel *et al.*, 1982). It stimulates both chemotaxis and mitogenesis in fibroblasts (Seppa *et al.*, 1982; Grotendorst *et al.*, 1984) and smooth muscle cells (Grotendorst *et al.*, 1982; Rutherford

& Ross, 1976). PDGF also stimulates the synthesis of collagen (Ross, 1987), fibronectin and hyaluronan (Blatti *et al.*, 1988; Heldin *et al.*, 1989), in addition to stimulating collagenase activity (Bauer *et al.*, 1985). The concentration of PDGF in an area determines what cells are most responsive to it; different cells being attracted by different concentrations of PDGF (Deuel & Huang, 1984). Although it strongly influences inflammatory and mesenchymal cell function, PDGF has no apparent direct effect on epithelial or endothelial cell function (Deuel, 1987).

D) Transforming growth factor beta - TGFβ

The TGFβ superfamily has been rerviewed by Massague, (1990). Three mammalian isoforms β1, 2 and 3, have been identified. The prototype member of the TGFβ family is TGFβ1, a 25Kd disulphide linked dimer of 2 identical 112 amino acid polypeptides (Derynck *et al.*, 1985). TGFβ has been isolated from a number of tissues including platelets, macrophages, lymphocytes (Sporn *et al.*, 1987), bone and kidney and it appears to be nearly ubiquitously produced (Lyons & Moses, 1990). TGFβ is found in high concentrations in the alpha granules of platelets and is released during degranulation at the site of injury (Assoian & Sporn, 1986). Regulation of TGFβ is controlled by macrophages in an autocrine manner (McCartney-Francis *et al.*, 1988). In addition, it stimulates monocytes to secrete other growth factors including FGF, PDGF, TNFα and IL-1 (McCartney-Frances *et al.*, 1988; Wahl *et al.*, 1987). Though generally associated with inflammation, it has the ability to down-regulate inflammatory activity through its influence on macrophage function.

Essentially all cells have receptors for TGFβ (Wakefield *et al.*, 1987) and should theoretically respond to it. The action of TGFβ appears to be carefully regulated. Active

TGFβ's exert their biological effects by binding to different types of cell surface receptors. In most cases three different types of receptors are observed, type I, II, III (membrane bound proteoglycan). In addition, TGFβ can bind to several soluble (α-2 macroglobulin, fibronectin, type IV collagen and thrombospondin) and membrane bound proteins which are not directly involved in signal transduction (Miyazono *et al.*, 1993). TGFβ is chemotatactic for macrophages (Wiseman *et al.*, 1988) and stimulates fibroblast chemotaxis (Postlethwaite *et al.*, 1987) and proliferation (Roberts *et al.*, 1985; Assoian *et al.*, 1984). In different concentrations it can either stimulate or inhibit cellular proliferation and its effect may be modulated by the presence of other growth factors. TGFβ1, 2, and 3 are present in a distinct temporal and spatial pattern in both normal skin and excisional wounds which suggests divergent functions for these molecules during cutaneous wound repair (Levine *et al.*, 1993; Schmid *et al.*, 1993). TGFβ's have been found extracellularly within hours of wounding, in association with inflammatory cells (Sieweke *et al.*, 1990) and migrating keratinocytes at the wound site (Kane *et al.*, 1991; Levine *et al.*, 1993).

TGFβ may be the most potent stimulant of collagen synthesis (Massague *et al.*, 1987; Sporn *et al.*, 1986; Roberts *et al.*, 1986) and in addition, it decreases the stimulatory effect of other factors on collagenase activity (Overall *et al.*, 1989; Edwards *et al.*, 1987). Fibronectin and proteoglycan synthesis by fibroblasts is stimulated by TGFβ (Ignotz *et al.*, 1987; Bassols & Massague, 1988) and fibronectin synthesis by keratinocytes (Wilkner *et al.*, 1988). TGFβ has the ability to organise matrix and may be involved in scar remodelling (Fukamizu & Grinnell, 1990). Endothelial cell proliferation is inhibited by TGFβ alone, but with a cofactor it functions as a stimulant

of angiogenesis *in vivo* (Roberts *et al.*, 1986; Folkman & Klagbrun., 1987). It can also stimulate epithelial cell proliferation (Coffey *et al.*, 1988).

TGF β has been shown to accelerate healing in both normal (Mustoe *et al.*, 1987; Ksander *et al.*, 1990; Beck *et al.*, 1990; Quaglino *et al.*, 1991; Pierce *et al.*, 1992) and impaired healing in animal models (Curtsinger *et al.*, 1989; Pierce *et al.*, 1989a; Beck *et al.*, 1991; Bernstein *et al.*, 1991; Cox *et al.*, 1993). Depletion studies (Shah *et al.*, 1992) suggest that TGFβ plays an important, but not indispensible role in normal wound repair, however the role of TGFβ or its isoforms in chronic wounds is unknown.

High levels of several growth factors, including TGFα, IGF I and TGFβ, in wound fluid from acute wounds, were found to stimulate wound cell migration (Banda *et al.*, 1982), proliferation (Greenberg & Hunt, 1978; Katz *et al.*, 1991) and regeneration of extracellualar matrix (Jalkenen *et al.*, 1983). In contrast, fluids collected from chronic wounds had low levels of peptide growth factors and failed to stimulate DNA synthesis in fibroblast cultures, suggesting deficiencies in growth factors may play a role in preventing the healing of chronic wounds (Schultz *et al.*, 1991). The addition of exogenous TGFβ2 to patients with non-healing venous ulcers resulted in a significantly faster healing rate by stimulating the formation of new granulation tissue (Robson *et al.*, 1993). This suggests that exogenous growth factors are inactive or at suboptimal concentrations in chronic wounds. These growth factors may be being degraded by proteases.

1.2.7 Proteases

Damaged and necrosed tissue must be removed before repair can commence. A delicate balance must be struck between matrix deposition and tissue turnover in wound

healing. It is likely that the association between proteolytic enzymes and their inhibitors contributes to this balance. Neutrophils and macrophages debride necrotic tissue by secreting proteases which accumulate in the extracellular fluid. Protease secretion, activation and expression is strictly regulated and involves a multistep conversion of the secreted inactive form of the enzyme (zymogen) into an active form which is then controlled by a variety of stimuli such as cytokines and tissue and blood inhibitors.

There are several different families of proteases; serine proteases, metalloproteinases and cathepsins. The serine proteases, including plasmin and plasminogen activator, degrade a wide range of extracellular matrix components and can activate collagenase (Werb *et al.,* 1977). There are seven well characterised metalloproteases, including interstitial and neutrophil collagenase, gelatinase and stromelysins. Cathepsins are lysosomal enzymes which actively degrade ECM components optimally at low pH.

A) Matrix metalloproteinases

The matrix metalloproteinase (MMPs) are a family of zinc and calcium-dependant endopeptidases that have the combined ability to degrade the various components of connective tissue matrices (Docherty and Murphy, 1990). Regulation of the MMPs is stringent, occuring not only at the level of gene expression but extracellularly, after secretion, by the action of activators of the proenzyme forms and of specific inhibitors. The major natural inhibitor, tissue inhibitor of MMPs (TIMPs), which is produced by the same cells, has been identified in two forms.

Nine MMPs have been identified by cDNA cloning and sequencing. Comparison of these sequences have revealed that two forms of collagenase, two gelatinases (type IV

collagenases), and two stromelysins occur. Three other enzymes, pump (punctuated metalloproteinase, matrilysin), "stromelysin-3" and metallo-elastase have different sequences and properties (Table 2).

The interstitial collagenases are the most specific of the MMPs, cleaving the native helix of the fibrillar collagens (types I, II, and III) at a single locus. MMP1 is produced by most connective tissue cells whereas MMP8, is confined to neutrophil granules (Whitman *et al.,* 1986; Hasty *et al.*, 1990). The gelatinases degrade types IV, V, VII, and X collagens and may act synergistically with interstitial collagenases in the degradation of collagens, since they efficiently degrade their denatured gelatin forms. They also degrade elastin. Two separate but similar cDNAs encoding a 72-kD gelatinase A (MMP2) and a 95-kD gelatinase B (MMP9) have been identified to date (Table 2), and the corresponding proteins have been shown to be associated with monocytes/macrophages, as well as, many stimulated connective tissue cells (Collier *et al.*, 1988; Wilhelm *et al.*, 1989).

Table 2 The matrix metalloproteinase family

MMP No	Trivial Name	kD	Source	Substrates
1	Interstitial collagenase	55	Connective tissue cells	Fibrillar collagens I, II, and III, collagen X, gelatin and proteoglycans
8	Neutrophil collagenase	75	Neutrophils	Fibrillar collagens, gelatin and proteoglycans
3	Stromelysin-1	57	Macrophages, connective tissue cells	Proteoglycan; cross-link regions of collagens II, IV, and IX; collagens X and XI; procollagens; fibronectin; laminin; gelatin; collagenase; gelatinase B
10	Stromelysin-2	57	Macrophages	As stromelysin-1 but much lower activity
2	Gelatinase A	72	Most cell types	Denatured collagens; collagens IV, V, VII, X, and XI; elastin
9	Gelatinase B	95	Monocytes, connective tissue, tumour cells	As gelatinase A
7	Pump (matrilysin)	28	Immature monocytes, mesangial, tumor cells (not fully defined)	As stromelysins, elastin
11	"Stromelysin-3"	51	Stromal cells of tumours	Unknown
12	Metallo-elastase	57	Macrophages (mouse)	Elastin, fibronectin

The stromelysins 1 (MMP3) and 2 (MMP10) are potentially very important members of the MMP family, having broad pH optima and substrate specificities. They are able to degrade many extracellular matrix proteins, including proteoglycans and laminin, and are also potentiators of collagnease and 95-kD gelatinase activity (Murphy *et al.*, 1987; Nagase *et al.,* 1991). Punctuated metalloproteinase (pump, MMP7, matrilysin), appears to be distantly related to the stromelysins but lacks the C-terminal domain. Pump has broad proteolytic activity that includes elastin and proteoglycans (Quantin *et al.*, 1989) and has been shown to be associated with immature monocytes (Busiek *et al.*, 1992). Stromelysin-3 has recently been found in the stroma of tumours, the trophoblast and fetal lung fibroblasts but no specific catalytic activity has yet been defined for this protein.

The most recently defined MMP cDNA is that for mouse macrophage metallo-elastase (Shapiro *et al.*, 1992). This enzyme could potentially be important in matrix turnover since it degrades many matrix macromolecules, including elastin.(Banda and Werb, 1981).

B) Tissue inhibitors of Metalloproteinases

The major physiologic inhibitor of the MMPs is α_2-macroglobulin, which is restricted in its sites of activity due to its large size (780 kD) and a family of inhibitors that are specific for the MMPs and are produced by many cell types, including those of connective tissues. Four inhibitors have been reported and two have been fully characterised and cloned. These TIMPs have closely related structures and inhibitory properties. TIMP-1 is a 30-kD glycoprotein (Docherty *et al.*, 1985) and TIMP-2 a 23-kD unglycosylated protein (Boone *et al.*, 1990). Their cellular distribution appears to be very

similar, although TIMP-1 is generally found in larger amounts. The occurrence of TIMP-2 is, however not yet fully documented. The TIMPs act specifically against the active forms of MMPs, TIMPs-1 and -2 having very similar activities (Ward *et al.*, 1991a) and forming essentially irreversible complexes. The mechanism of TIMP inhibition is not fully understood.

C) Extracellular regulatory mechanisms

The regulation of MMPs is necessarily stringent and in most cases occurs at the level of gene expression. A second level of regulation occurs extracellularly and is thought to be of some importance, ultimately controlling the level of enzyme activity in terms of matrix destruction, the activation of secreted proenzyme forms and their subsequent inhibition of TIMPs (Docherty & Murphy, 1990; Docherty *et al.*, 1992). The action of potential physiologic enzyme activators, which include plasmin, plasma kallikrein, cathepsin B, cathepsin G and neutrophil elastase, have been analysed (Nagase *et al.*, 1991). Plasmin has long been thought to be an activator of MMPs and a number of studies using cell model systems with connective tissue and tumour cells have been conducted (Murphy *et al.*, 1992). Stromelysin-1, which can be sequestered on the collagenous matrix, is particularly susceptible to plasmin activation and once activated can potentiate collagenase activity and act as a gelatinase B activator. TIMPs may regulate the activation process to some extent, since it has been shown that they can slow down or prevent the autocatalytic cleavages that occur after initiation of activation by enogenous proteinases (Murphy, *et al.*, 1992).

Gelatinase A differs in its mechanism of extracellular activation from other

MMPs. The propeptide of this enzyme has no apparent cleavage site susceptible to plasmin and other proteases, although it can undergo self-cleavage reactions to lose the propeptide and become active (Nagase *et al.,* 1991; Ward *et al.*, 1991b). However, this enzyme can be activated by a fibroblast or tumour cell membrane-mediated process that is sensitive to metalloproteinase inhibitors. The membrane activator which is specific for gelatinase A and does not activate the other pro MMPs (Ward *et al.*, 1991a) can be induced by a number of effectors including concanavalin A, phorbol ester and transforming growth factor-β (Ward *et al.*, 1991a). It is not known if the activator is a proteinase or merely binds gelatinase A and initiates autocatalytic cleavages. This activation is efficiently prevented by TIMP-2 when bound to the proenzyme but not by TIMP-1, which only binds to active gelatinase A (Murphy *et al.*, 1992; Ward *et al.*, 1991b). Gelatinase A is a widespread proteinase and appears to be expressed by many cells. It is feasible therefore, that the membrane activation mechanism plays an important regulatory role, by controlling the precise locations at which this enzyme may be activated.

In many cell model systems, the ability of TIMPs to prevent matrix degradation has been demonstrated (Moscateli and Rifkin, 1988). TIMPs have been shown to be sequestered in tissues such as cartilage but no evidence of direct matrix binding has been obtained. Like plasminogen activators, they may be limited in their action by the focal nature of enzyme activation at certain sites on the cell surfacc.

Overproduction of these proteases in chronic wounds may lead to excessive degradation of pre-existing matrix molecules, adhesion proteins, cytokines and their receptors (Grinnell *et al.*, 1992) as well as the new extracellular matrix molecules which

are formed during fibroplasia.

1.3 EQUINE WOUND HEALING

Skin is a complex organ that cannot regenerate and therefore must repair itself after injury (Silver, 1982). In normal circumstances, wound defects are replaced by fibrous tissue and surfaced with epithelium to re-establish the continuity of the skin. However, wound healing in the equine species can be significantly delayed and more complicated than in other species. Wounds on the lower limbs are particularly liable to slower healing and further more, show a higher incidence of complications than wounds on other parts of the body. These wounds that have many similarities to the chronic skin wounds encounted in human medicine. Chronic wounds differ in nature but the common conditions that can lead to these include: arteriosclerosis, diabetes mellitus, pressure, venous insufficiency, and steroid hormone use (Hunt, 1991). It is generally thought that overall macrovascular and microvascular problems contribute to the impaired healing. Several signs of non-healing may occur, such as the presence of necrotic tissue, excess fibrotic or granulation tissue. There may also be various types and large numbers of pathogens, changes in pH, or oxygen gradients and the quality of cellular exudate which will impair wound healing. Normal wound healing routinely occurs in sites other than the distal limb of the horse and lesions re-epithelise swiftly often with minimal wound care. Equine chronic wounds are a major problem for the horse-owning community, unsightly wounds are not acceptable on horses with commercial potential e.g. showjumpers or racehorses and they are frequently responsible for restriction of performance and loss of commercial value.

Equine wound healing has not been studied in great detail, far more emphasis has been placed in the investigation of the healing of chronic human wounds. It is possible that equine wounds may make a suitable naturally occurring wound model for chronic wound healing in man. In this study equine wound healing has been compared to the problems encountered in man which have similarities e.g. chronic skin ulcers (man) and chronic indolent wounds (horses).

1.4 NON-HEALING WOUNDS

1.4.1 EXUBERANT GRANULATING WOUNDS

Granulation tissue formation is a necessary component of wound repair. One of its primary functions is to accumulate macrophages and fibroblasts which facilitate the deposition of loose connective tissue and angiogenesis. It also provides a barrier against external contaminants. Granulation tissue can prevent effective wound healing, by obstructing epithelial migration and wound contraction if it becomes excessive or exuberant. Plate 1.1 shows an example of a horse with exuberant granulation tissue formation on a non-healing wound. Excessive granulation tissue formation does not usually impair wound healing in other domestic species (Bertone 1989b). Horses however, tend to produce exuberant granulation tissue to a greater extent and much earlier after wounding especially in the lower limb regions especially below the carpus and stifle (Fretz *et al.*, 1983; Jacobs *et al.*, 1984; Bertone *et al.*, 1985; Blackford *et al.*, 1986; Bertone, 1988; Lee and Swain, 1988; Lindsay 1988). Wounds on the lower limbs produce noticeably different looking granulation tissue than wounds in other areas and commonly heal more slowly than wounds on other parts of the body (Jacobs *et al.*, 1984).

Unsightly exuberant granulation tissue is formed, which affects the welfare of horses is socially unacceptable and often delays or halts wound healing. Secondary complications may arise e.g. bacterial infections, trauma or phycomycoses (Bertone, 1989b).

Wound management protocol such as topical medications, bandaging, casting and immobilisation may influence excessive granulation tissue formation. There are several possible explanations for the relative difficulties of equine limb wound healing, these are:

1) increased movement
2) increased skin tension
3) reduced vascularity
4) thinness of skin
5) lower temperature
6) higher susceptibility to infection
7) lack of appreciable underlying muscle tissue
8) reduced oxygen tension
9) pH alterations

(Bertone, 1989 a & b; Britton , 1970; Hogle *et al.*, 1959; Jacobs *et al.*, 1984).

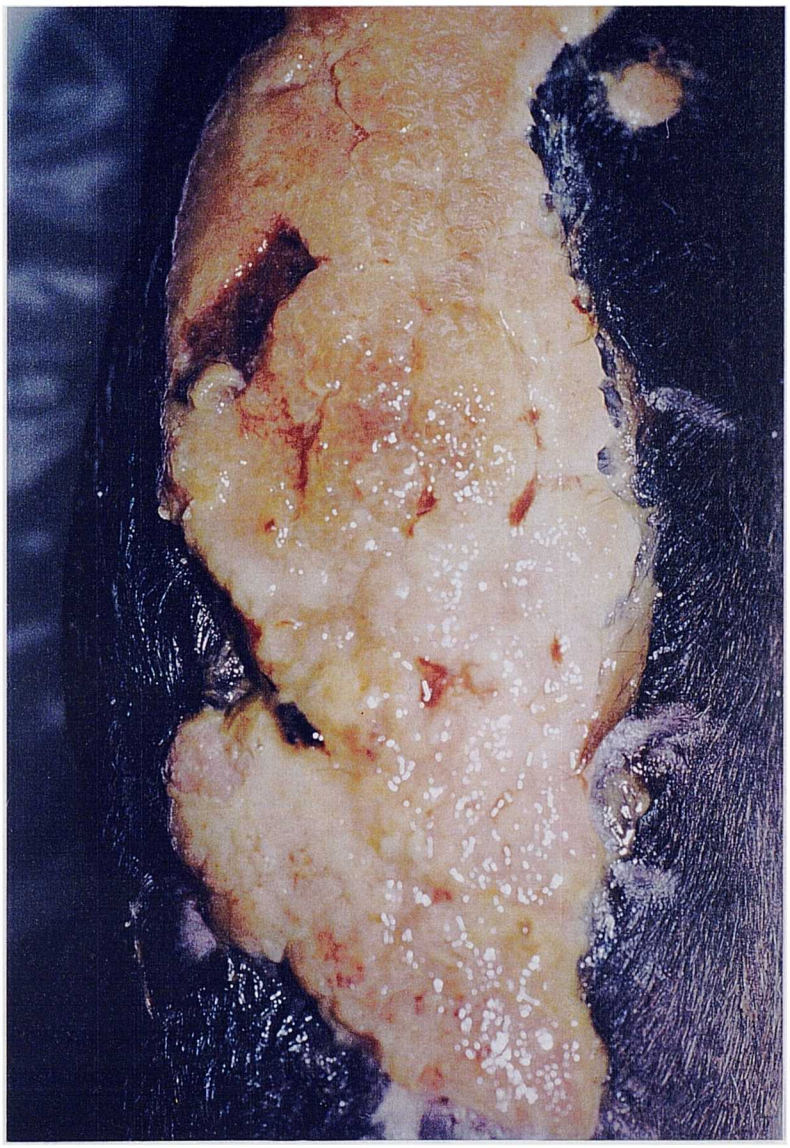


Plate 1.1 A chronic wound on the distal limb of a horse showing exuberant granulation tissue formation.

1.4.2 INDOLENT NON-HEALING WOUNDS

Plate 1.2 shows the other type of non-healing equine wound, the indolent wound. This type is very similar in clinical appearance to some chronic wounds in humans (eg. venous leg ulcers). It is a chronic wound which remains unhealed for long periods of time, possibly up to several years. Many chronic skin ulcers in man are due to underlying venous disease which may also be a factor in horses, but has yet to be investigated. Ambulatory venous hypertension (failure of venous blood pressure to fall during exercise) results in drastic cellular changes leading to leg ulcer formation (Sarin *et al*, 1991; Shami *et al*, 1992), this has not been investigated in the equine species but may be of benefit in understanding equine chronic wound healing. It is thought that venous insufficiency is the result of a number of microvascular changes in the skin, e.g.dilated capillaries often surrounded by fibrin, oedema, decreased fibrinolysis, white blood cell trapping and heterogenous perfusion leading to tissue ischaemia and hypoxia.

Browse and Burnard (1982), proposed that as a result of ambulatory venous hypertension, large molecules such as fibrinogen leak out through enlarged pores of damaged and distended dermal blood vessels. Fibrinogen is quickly polymerised to an insoluble layer of fibrin and produces cuffs around individual capillaries. These cuffs are thought to impair the exchange of gases and nutrients between the plasma and dermis leading to tissue anoxia and ulceration. Venous ulcers are located only on the lower extremities of man and this corresponds with the location of indolent wounds in horses. In man, it is well documented that these conditions usually develop in the elderly over the age of 60 years. In the horse, however age does not appear to have any significant effect on the development of a non-healing wound on the lower limb.

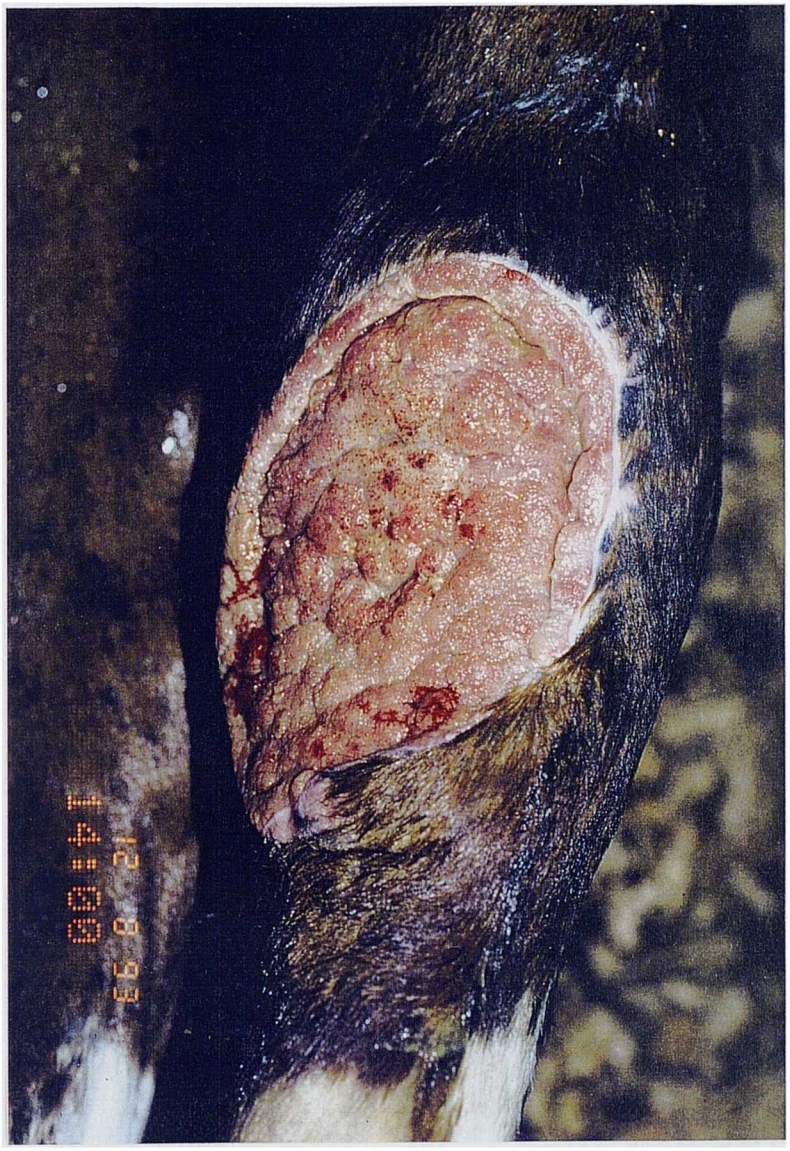


Figure 1.2 A wound with an indolent, non-granulating bed showing little or no evidence of either epithelisation or granulation.

The chronic indolent wound produces very little or no granulation tissue and therefore, it is impossible for epithelisation to take place as the epithelial cells are unable to migrate over the wound surface.

Granulation tissue deposition in these wounds is thought to occur in an ordered sequence. Initially deposition of fibronectin and hyaluronic acid is followed by collagen type III and finally by collagen type I (Gay *et al*, 1978; Kurkinsen *et al*, 1980). The modulation of cell behaviour is thought to be controlled independently by cytokines and the extracellular matrix. The extracellular matrix is altered by cells depositing new matrix molecules and degrading the existing matrix. Fibroblasts and other cells deposit cytokines into the extracellular matrix, which acts as a reservoir for these substances. The matrix binds to specific cell surface receptors which facilitates attachment, migration and proliferation. Collagen and fibronectin play an important role in normal wound healing although, their role in chronic wound healing is not known. Any disruption of production or degradation may contribute to the wound healing problems incurred in equine wound healing.

1.4.3 TREATMENT

Granulation tissue has historically been managed by the use of:

1) various chemical cautery agents e.g. copper sulphate, silver nitrate etc.

Unfortunately caustic chemicals are also toxic to migrating and proliferating epithelium and therefore epithelisation is often simulataneously arrested with such treatment.

2) Surgical resection is also used to control exuberant granulation tissue. Strips of granulation tissue can be shaved from wound beds to produce a flat surface which is level with the surrounding edges. The epithelial margin should be preserved to allow continued healing, followed by bandaging.

3) Cast application protects wounds in highly mobile areas and commonly provides rapid, cosmetic wound healing and is effective for wounds of the lower limb.

1.4.5 SKIN GRAFTING

One of the most effective treatments is the use of pinch grafting. Pinch grafting appears to inhibit fibroblast proliferation in healthy granulation tissue. Skin grafts expand in the granulation tissue, decrease healing time and therefore, prevent the formation or reformation of exuberant granulation tissue. Seed grafts are easy and relatively inexpensive to apply (Schumacher & Hanselka, 1989). Pinch grafts of split thickness that are composed of both epidermis and dermis are usually taken from the neck of the horse and embeded into a granulation bed which has been carefully prepared by debriding as much unhealthy granulation tissue as possible (Schumacher & Hanselka, 1989). The harvested grafts consist of thin, intermediate and full-thickness grafts placed in granulation tissue pockets at specific intervals (usually 0.75-1.0 cm apart) in the granulation tissue bed (Hogle *et al*, 1959; Frankland *et al*, 1976). These pockets provide an excellent protective nutritive environment that is ideal for graft acceptance. As the epithelial cells grow from the graft, the cover of granulation tissue over them begins to blanch and become paler in colour which suggests that angiogenesis is being

switched off or otherwise altered in the granulation bed (Hogle *et al.*, 1959; Schumacher & Hanselka, 1989). The grafts appear to stimulate the growth of epithelium from the wound edges as well as, providing a source of epithelium that spreads from the edges of the graft over the granulating bed until the epithelial zones merge (Schumacher & Hanselka, 1989). Proliferation of granulation tissue is noticeably inhibited once the grafts are viable. Grafts are obtained by elevating the donor tissue with a vecole, after which they are cut as thin as possible at right angles to the elevation, so that grafts 2 to 3 mm in diameter are obtained (Plate 1.3). The grafts are then flattened and inserted with their epithelial surfaces outward into the granulation tissue pockets. This process is continued until the entire wound surface is seeded with grafts. Sterile non-adhesive and moist dressings and bandages are applied to the grafted wound and it is left undisturbed for four to seven days. After care consists of rebandaging and cleansing of the wound at four to seven day intervals until graft takes are complete (usually 28-34 days). After this time dressings are removed and it can be seen that repithelialisation has occurred around the vicinity of the graft (Plate 1.4). There are several advantages of skin grafting:

1) grafts are easy to apply

2) they are relatively resistant to infection

3) they can withstand movement.

The disadvantages include:

1) poor cosmetic end result, which leaves a cobblestone appearance with tufts of hair sprouting from the grafts

2) poor quality of skin healing that may bleed and crack with movement (Meagher & Adams, 1971).

In most cases, a 50 to 60% graft take can be expected and the donor sites heal cosmetically. Epithelisation of the wound is usually obvious by 50 to 65 days, when hair is starting to show through the wound surface as shown in Plate 1.5. It may be necessary to perform this procedure on several occasions before the wound closes entirely (Hogle *et al.*, 1959; Schumacher & Hanselka, 1989. The cosmetic effect is not commonly very satisfactory but the main priority is to close the wound.

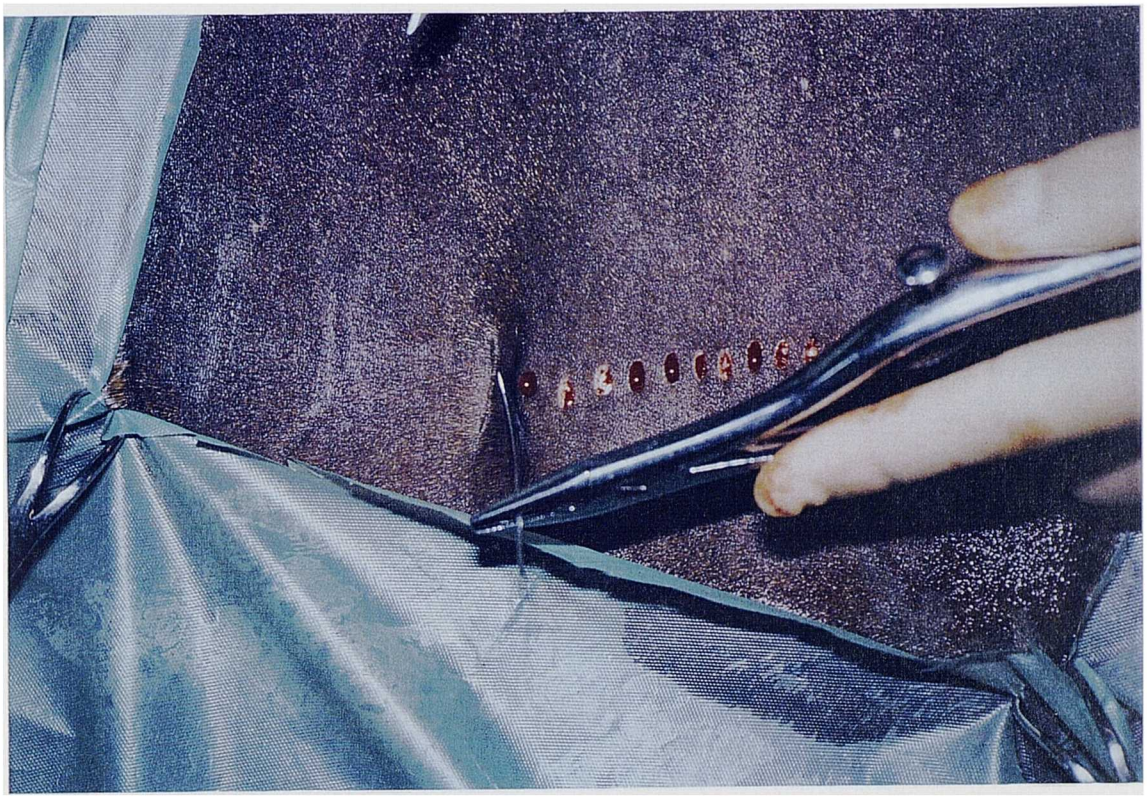


Plate 1.3 Pinch grafts are removed from the donor site of the horse (usually the neck).

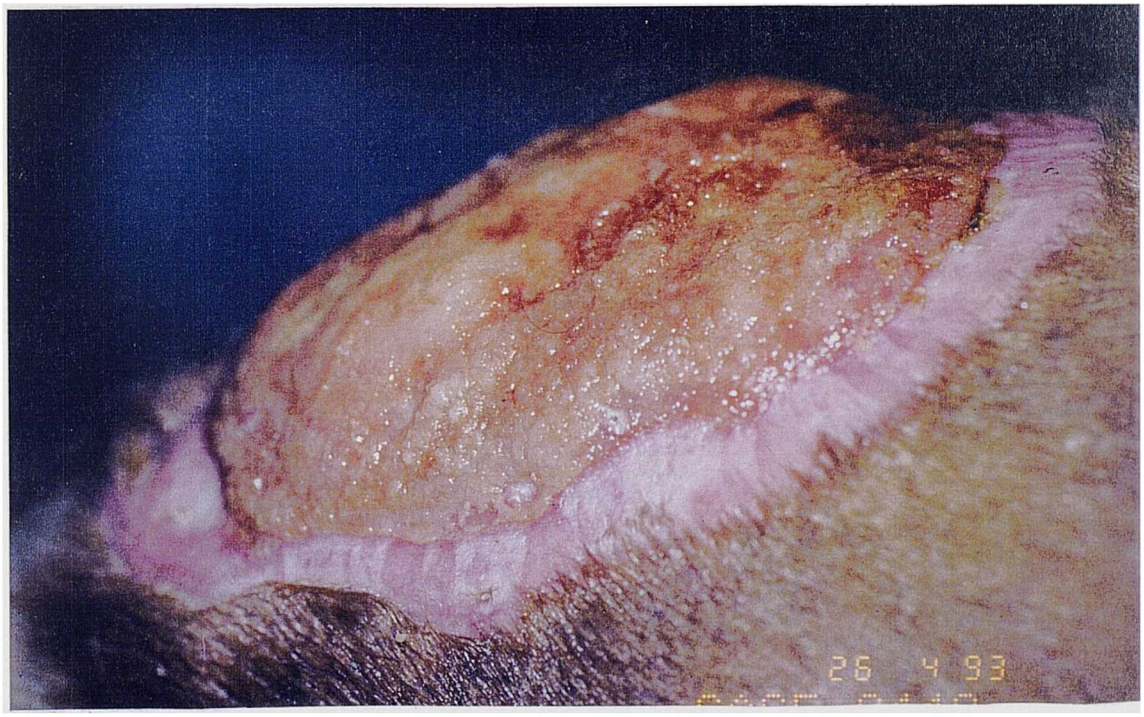


Figure 1.4 Reepithelisation occurs in the viscinity of the grafts and at the wound margins

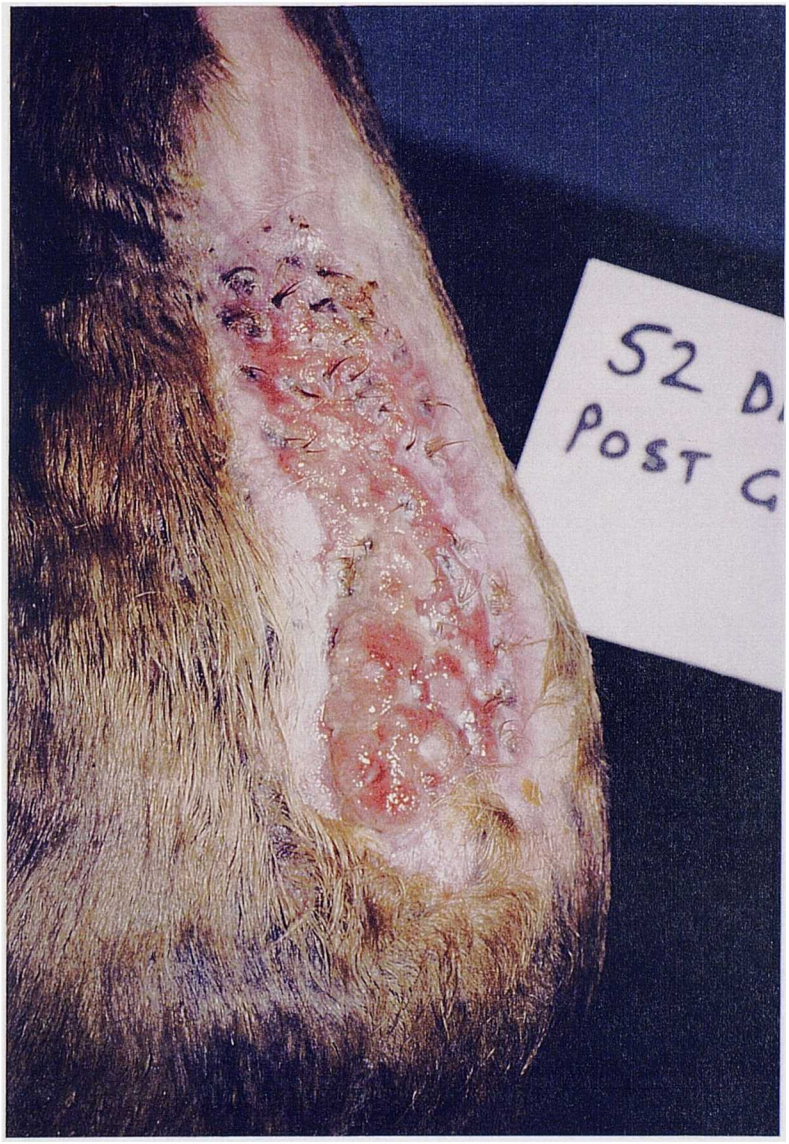


Plate 1.5 Wound on the distal limb of a horse, 52 days post grafting. Notice the appearance of hairs in the wound area.

After grafting, it may be necessary to apply rigid casts over wounds in highly mobile areas such as the lower limbs (Bertone, a & b, 1989; Schumacher & Hanselka, 1989).

1.5 SARCOIDS

Sarcoids are locally invasive tumour-like lesions of the skin with a high propensity for recurrance. They are the most commonly diagnosed skin tumours of equidae (Jackson, 1936) and is probably the most common cutaneous reason for euthanasia (Knottenbelt *et al* 1995). It is not uncommon for sarcoids to appear at the site of wounds, as shown in Plate 1.6. Sarcoid lesions may be found on all parts of the body but they are least common on the dorsum of the trunk. Sarcoids are identified by histopathological classification and such confirmation is often necessary to distinguish the fibroblastic form of sarcoid from exuberant granulation tissue, other rare mesenchymal tumours, other infectious and non-infectious granulomas (Genetzky *et al.*, 1983; Tarwid *et al.*, 1985; Adams *et al.*, 1988). It is not known if the sarcoid is truly neoplastic or simply an abnormal epithelial and fibrous proliferation (Gorman, 1985). There is circumstantial evidence that supports the idea that it is caused by a virus. This could possibly be a retrovirus or a virus related or identical to the bovine papilloma virus. There are six different forms of these lesions and they are all distinct from each other, many lesions will have overapping characteristics.

Occult sarcoid

The superficial epidermal layers are affected with this type of sarcoid. They appear as circular areas of alopecia with a grey, scaly surface. They commonly appear on the medial thigh, sheath, neck and face but may be found in almost any area of the body.

Verrucous sarcoid

These sarcoids are seldom localised and have an ill-defined margin. The surface has a rough appearance with irregular thickened hyperkeratotic areas and flat, scaly areas (Knottenbelt *et al.,* 1995). Location is most often in the axilla and groin and occasionally the side of the face, the ear base and the breast.

Nodular sarcoid

Nodular sarcoids are usually subcutaneous and have the appearance of spherical nodules under apparently normal skin. The overlying skin commonly has an almost transparent shiny appearance under which the lesion is attached. The lesions may vary in size, number and distribution. They are most commonly found in the groin, thigh and on the eyelids.

Fibroblastic sarcoid

These are the most "normal" aggressive type of sarcoid. The surface is usually ulcerated and is liable to trauma, haemorrhage and local infection. The lesions can develop at almost any site and represent a common complication of skin wounds, particularly of the limbs.

Mixed sarcoid

This form of equine sarcoid consists of two or more types of lesions and is most commonly encountered in the axillae, groin and around the face.

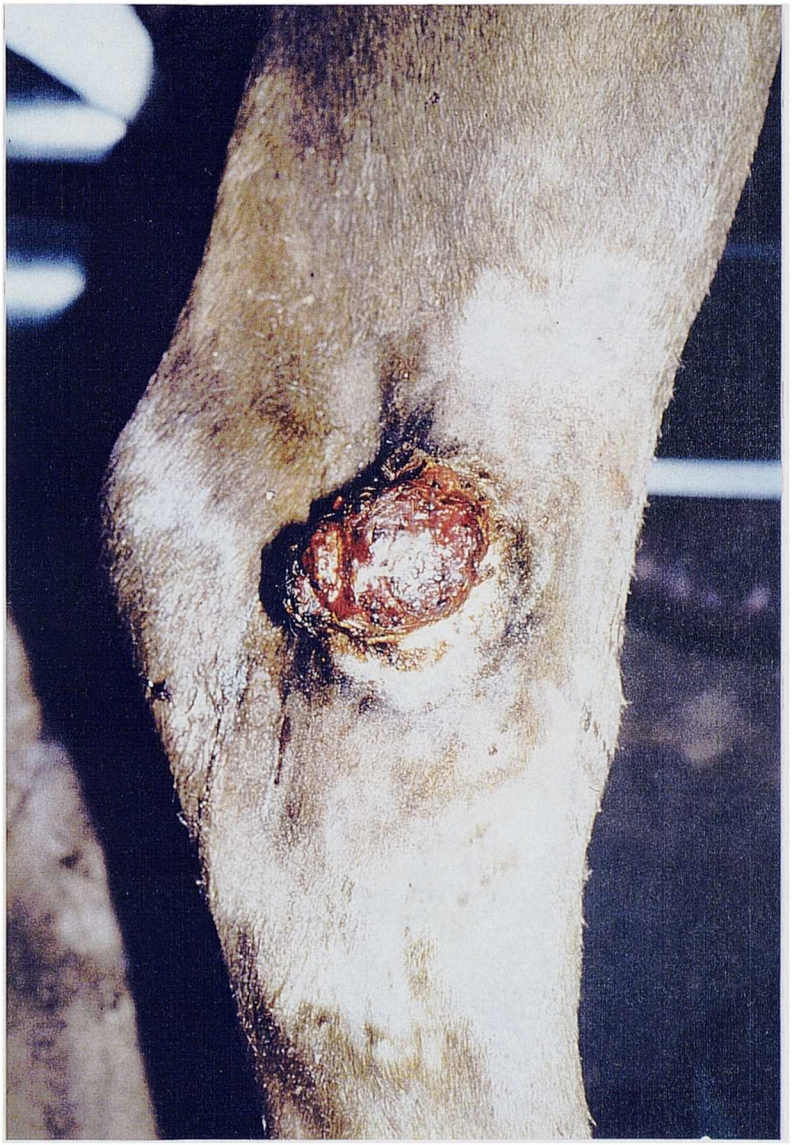


Plate 1.6 A wound complicated by the development of an equine sarcoid tumour.

Malevolent sarcoid

They are particularly invasive sarcoid tumours which infiltrate lymphatic vessels resulting in multiple tumour masses along these vessels and at remote sites such as local lymph nodes (Knottenbelt *et al.,* 1995). The malevolent form usually follows interference with a fibroblastic mass, at the elbow or jaw and rapidly extends to produce cords of tumour with nodules occurring at irregular intervals along their length.

1.5.1 PATHOLOGICAL FEATURES

Sarcoid tumours characteristically show linear or focal dermal thickening which is pale in colour and firm in texture, due to the fibroblastic proliferation and the relatively few capillaries contained within the lesion (Willams *et al.,* 1982). Histological diagnosis is based on the presence of a fibroblast proliferation of moderate to high cell density with few capillaries and a low mitotic index. Individual cells are spindle-shaped, forming whorls, interlacing bundles and haphazard arrays with one another (Plate 1.7). Cell morphology varies greatly and as in most mesenchymal tumours the cytoplasmic boundaries are ill-defined. Metastasis does not occur, but there are reports of the development of multiple, small lesions following incomplete surgical removal of one or more sarcoids or after the administration of autogenous vaccines.

1.5.2 EPIDEMIOLOGY

Sarcoid tumours have been reported in almost all horse breeds, although noticeable differences exist between breeds. Quarter Horses had almost twice the risk of developing sarcoids as Thoroughbreds, while Standardbreds had a much lower risk of developing sarcoid relative to all other breeds (Marti *et al.*, 1993).

Sarcoids seen in the UK have some noticeable differences from those seen in some other parts of the world (Knottenbelt *et al.,* 1995). Horses in Australia, for example are less likely to have large numbers of sarcoids and horses from Europe and North America may show an average of between two and eight sarcoids per horse. In the UK however, the numbers may be between ten and several thousand per horse. There is strong circumstantial evidence that flies are involved in the pathogenesis and epidemiology of the disease, possibly different biting flies in different geographical areas have some role to play in the regional variations in numbers and types of sarcoids (Knottenbelt *et al.,* 1995).

The earliest suggestion that sarcoid may have an infectious origin came from studies by Olsen (1948) and Olsen and Cook (1951), who demonstrated that intradermal inoculation of cell-free extracts from bovine skin tumours containing bovine papilloma virus (BPV) into horses caused lesions that resembled equine sarcoids. These observations were later confirmed (Ragland and Spencer 1968, 1969). A limited number of sarcoid tissue extracts reproduced the disease when injected into other sites on the donor animal's body or other horses (Voss, 1969). In naturally occurring cases neither spontaneous resolution nor antibody to any of the BPV variants have been found.

Molecular hybridisation techniques revealed BPV DNA sequences in the vast

majority of sarcoid lesions (Lancaster *et al.*, 1977, 1979; Trenfield *et al.*, 1985). The first of these studies revealed that there was either only a portion of the viral genome present or that the BPV-DNA in these lesions was derived from a number of closely related viruses. Later research demonstrated the BPV-like DNA in equine sarcoids is not integrated into the host cell (Lancaster, 1981). BPV sequences in equine sarcoids occasionally exhibit restriction enzyme cleavage patterns different from those predicted from the nucleotide sequence of the prototype viruses (Angelos *et al.*, 1991) raising the possibility that there may be variants to BPV that are specific for horse arising through mutational events. BPV has the ability to transform fibroblasts *in vitro* (Wood and Spradbrow, 1985). However, to date no virus particles have been demonstrated in any sarcoid (Reid *et al.*, 1994).

1.5.3 THERAPY AND RESPONSE OF EQUINE SARCOIDS

There are a limited number of therapeutic options available to the veterinary surgeon and there has been little progress made either in the treatment or control of these sarcoid lesions. If there are only a few lesions, they do not interfere with normal function or if the condition is considered to be too severe, it is apparently better not to interfere with them therapeutically.

Several types of therapy are open to the veterinary surgeon. These include:

Surgical removal

Surgical excision has been performed for many years, some surgeons have good results while others report problems. A major problem is the tendency for these lesions to regrow at the site. It is unlikely that this form of treatment is suitable for tumours on the eyelid or the distal limb, due to cosmetic effects, inability to close the resultant wound and interference with normal function (Knottenbelt *et al.*, 1995). Small well-defined tumours have the best prognosis for surgical removal while extensive areas of poorly defined verrucous and mixed sarcoid may result in rapid regrowth of a more aggressive sarcoid type. Sarcoid regrowth can occur at the site of previous surgery up to ten to fifteen years later. There is a distinct tendency for sarcoids to develop at the site of other skin injuries, including naturally occurring wounds and surgical wounds such as castration (Knottenbelt *et al.*, 1995). The most effective treatment of nodular sarcoid is by surgical excision, however surgical excision of nodular eyelid tumours is not recommended as they tend to develop deep and extensive local infiltration.

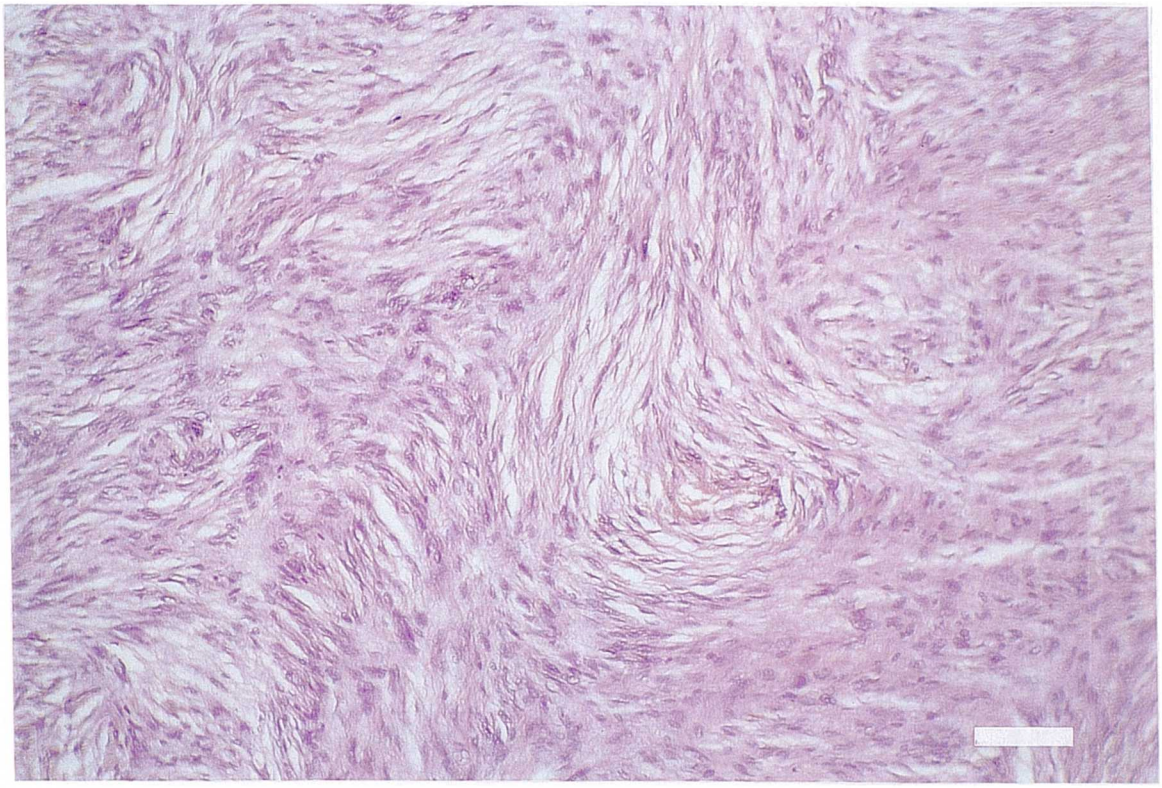


Plate 1.7 Histological features of sarcoid tissue. Notice the whorl formation of fibroblasts arranged in bundles. Section stained with H & E. Bar: 100μm.

Cryosurgery

In most cases surgeons using cryosurgery will debulk larger masses first and selection of lesions may bias the results in favour of high resolution. Lane (1977) demonstrated a 44% chance of sarcoid regrowth. Cryosurgery is not generally suitable for the eyelids or masses overlying joints. There are several drawbacks to the procedure, these include: prolonged general anaesthesia, careful placement of the thermocouples is necessary and the ability of the body to resist freezing. These make other methods more effective and desirable. Interestingly, Lane 1977 reported the regression of lesions remote from those subjected to cryosurgery but this has not been corroborted in other reports.

Hyperthermia

Radio-frequency current-induced hyperthermia has been reported to induce regression with no recurrence at 7-12 months after the last treatment in three cases of sarcoid (Hoffman *et al.*, 1983). Tumours require repeated treatments and the benefits are slow to develop.

Immunotherapy

Autogenous vaccines, which are commonly and effectively used in the treatment of the equine viral papillomata, have shown no consistant therapeutic effect. Both the

severity and the number of lesions may increase following their use.

Immunomodulators, such as BCG cell wall extracts of varying purity, have proved to be more effective. The mechanisms are unknown and the effects are limited to individually injected tumours. The ability to produce the desired effect relies upon and adequate relative volume of antigen being injected directly into the lesion and the response relies upon an effective immune capacity of the host, relatively few tumours and suitable formulation of the protein (Knottenbelt *et al.,* 1995). The practicality and cost of the technique has its limitations due to the fact that each tumour has to receive a defined amount of antigen repeatedly. Complications can arise from BCG therapy which include death from anaphylactic shock after two or more injections. It has been suggested that the use of BCG in the treatment of fibroblastic sarcoid on the lower limb and abdominal wall will aggravate the tumours. The best results are obtained in treatment of nodular and fibroblastic sarcoids (Marti *et al.*, 1993; Knottenbelt *et al.*, 1995).

Radiation therapy

Radiation brachytherapy (using gold ^{198}, radium^{226}or iridium ^{192} sources) have been used for the treatment of equine sarcoid. The efficacy of radiation is good but management and cost implications limit its use. The treatment has a major advantage in that scarring is minimal and the cosmetic results are very accepable.

Chemotherapy

A wide variety of topical cautery agents have been applied over the years to equine sarcoids, including sulphuric acid, nitric acid, silver nitrate, mercuric chloride, copper sulphate, arsenic trioxide, and several lead and antimony salts and various mixtures of these, podophyllum and 5-fluorouracil(Marti *et al.*,1993; Knottenbelt *et al.*, 1995). Trials are being undertaken at Liverpool University on a topical ointment, which has so far resulted in an overall 70% resolution rate (Knottenbelt & Walker, 1994; Knottenbelt *et al.*, 1995). The ointment is applied repeatedly, directly to the surface of sarcoid lesions and results in preferential necrosis and sloughing of the sarcoid tissue over a period of five to ten weeks.

1.6 Conclusions from the literature

As very little research into equine wound healing has been published, it has been necessary to compare chronic wound healing in the horse with similar wounds occuring in humans. The relationship between the microvascular problems of human chronic wounds and the effect these may have on the various stages of wound repair has been discussed. However, little is known about the microvascular circulation of the horse, therefore, many of these theories may be hypothetical. Understanding these complex events may lead to a better understanding of equine wound healing and sarcoid formation and recurrence which unfortunately is extremely common in horses.

The chronic wound environment may affect the ability of cells, especially fibroblasts, to synthesise matrix molecules, such as fibronectin and collagen. Insufficient or uncoordinated production of critical growth factors and the lack of vital metabolic substrates, such as oxygen, may lead to a dramatic impairment of the extracellular matrix and delayed wound healing (Schultz *et al*, 1991). The migration of fibroblasts may be inhibited due to reduced levels of chemoattractants or a defective ability of fibroblasts to respond to such stimuli. A wound bed lacking essential extracellular matrix molecules would limit resistance to infection, cell adhesion and migration, further matrix formation and tensile strength. Angiogenesis and epithelisation would not commence and the wound would not heal.

Normally, *in vitro* and *in vivo* animal models have been used in the study of wound repair. These investigations allow the isolation of specific wound events, the evaluation of assays and the examination of cell to cell/matrix interactions using a selected environment. However, these studies do not represent what is truly occuring in

the chronic wound, due to lack of correlation and relevance to the clinical model and to the elimination of clinical complications and the lack of replication of the chronic wound environment.

The horse can provide a suitable naturally occurring model of chronic wound healing. Initial wound formation, inhibitors of repair and features of the eventual healing process may be found through careful examination of the chronic non-healing wound in the clinical environment. Examination of the pathophysiology, etiology and wound environment, including the wound exudate, the inhibitors of healing and factors likely to aid healing may be identified. Tissue biopsies provide a means of quantitative and qualitative analysis of wound repair, which allows the investigator to compare chronic wound tissue with normal healthy skin. There have been no biochemical or *in vitro* studies using biopsy material derived from equine wounds or sarcoids. Such studies would be useful in understanding the mechanisms leading to the failure of wound healing whether in exuberant, indolent or sarcoid affected wounds in the horse.

1.7 AIMS OF THIS THESIS

It is clear from the literature review that very little is known about the process of wound healing in the horse or of the mechanisms whereby wound healing may be defective. It was therefore felt necessary to investigate both the basic structural and chemical changes in the equine wound, key aspects of the healing process, identified in humans, which may be defective in exuberant granulation tissue formation or in the occurrence of sarcoids. The hypothesis was that growth factors and extracellular matrix molecules may influence the character and rate of wound healing in the horse.

Specifically the aims of this study were:

1. To study the appearance and changes in tissue architecture of the equine wound during wound healing and sarcoid formation. Extracellular matrix molecules and growth factors were examined using histological, immunohistochemical and biochemical techniques.

2. To study the biosynthetic activity of normal skin, granulation tissue and sarcoid derived fibroblasts in response to growth factors and serum free media.

3. To study the behaviour of normal, granulation tissue and sarcoid-derived cells *in vitro* with the treatment of exogenous growth factors in respect of morphology, proliferation and collagen production.

4. To investigate the role of metalloproteinases and their inhibitors in chronic wound fluid, serum and tissue homogenates using biochemical techniques.

CHAPTER TWO - Methods

2.1 Histological and immunohistochemical studies

2.1.1 Source of tissue for histology and immunohistochemistry

Skin biopsies were taken from horses under general anaesthesia. Tissue was removed during the course of normal wound debridement prior to skin grafting. Table 2.1 shows the source of normal, granulation and sarcoid tissue selected. Two rectangular pieces of tissue, approximately 12mm long and 5mm wide were selected from the tissue excised by a veterinary surgeon. The position of the biopsy was standardised between horses and included epidermis, papillary, reticular and deep dermis.

Table 2.1 Source of tissue for histology, immunohistochemistry and biochemistry

TYPE OF TISSUE	BREED	SEX	AGE (years)
Normal	TB	Mare	15
	TB	Gelding	8
	TBX	Mare	12
Granulation	Palamino	Gelding	12
	TB	Gelding	5
	TBX	Filly	2
Sarcoid	TBX	Gelding	3
	TBX	Gelding	10
	TB	Gelding	5
	TB	Mare	4

2.1.2 Immediate treatment of biopsy

One half of each biopsy was fixed in cold 4% paraformaldehyde in phosphate buffered saline (PBS) and stored at + 4°C. The other half was placed within OCT compound (Raymond Lamb, London) in an embedding mould and snap frozen over liquid nitrogen in the operating theatre. The biopsies were carefully orientated within the block before freezing to ensure sectioning through the long axis of the specimen. The embedded specimens were then stored at -70°C for later immunocytochemical analysis.

2.1.3 Histological examination

After fixation the specimens were dehydrated through a series of alcohols, 1 hour in each of the following: 50, 70, 90 and 100%. The specimens were then cleared in 50:50 alcohol /chloroform for 30 mins and a further chloroform treatment for 1 hour. Specimens were dried and embedded in molten wax under vacuum at 60°C in paraffin wax. The wax blocks were sectioned at 4μm on a Bright 5030 microtome and sections floated onto slides in a water bath at 50°C to allow them to expand. The sections were allowed to adhere to the slides overnight at 40°C. Sections were stained with Haematoxylin and Eosin (H&E) and Massons Trichrome.

Sections were viewed on with an Olympus BH2 microscope and photographed with an Olympus PM-10AK photographic system using Kodak Ektachrome 64T Tungsten film from which colour prints were made.

2.1.4 Immunocytochemical examination

Frozen blocks were sectioned at 7μm using a Leitz cryostat and the sections were collected onto slides coated with 0.01% poly-L-lysine (Sigma). The slides were fixed in 100% acetone for 10 mins, air dried and stored at -20°C until required for staining. Primary and secondary antibodies used for indirect staining are shown in Table 2.2 and Table 2.3 respectively. Serial dilutions of each antibody were performed to determine the appropriate concentration for use. Cryosections were incubated in humidified chambers for 1 hour with primary antisera against extracellular matrix molecules and endothelial cell adhesion molecules. Staining with antisera against growth factors was performed at 4°C for 24 hours. The sections were washed (3x 5 mins) with PBS containing 1% BSA to block non-specific binding and then incubated with biotinylated secondary antibody for an hour at room temperature. After further washing the sections were incubated with FITC-conjugated streptavidin for 20 minutes at room temperature. This was followed by three, 5 minute washes with PBS (1% BSA) and the sections were mounted in Vectashield mounting medium (Vector laboratories). Control sections were stained by substituting the primary antibody with PBS followed by the above protocol. Stained sections were stored at 4°C and viewed before fading (within a week). The stained sections were visualised by epifluorescent microscopy using a Leitz Orthoplan microscope fitted with a Leitz Orthomat automatic camera and photographed using Kodak Ektachrome 400 film.

Table 2.2 Primary antibodies used for indirect immunofluorescence

Antibody raised against	Antibody raised raised in	Supplier	Dilution (In PBS)
Extracellular matrix molecules			
Laminin	Mouse	Sigma	1:100
Fibronectin	Mouse	Sigma	1:100
Collagen I	Rabbit	Bradsure Biologicals	1:1000
Collagen IV	Mouse	Dako	1:1000
Fibrinogen	Mouse	Chemicon	1:100
Endothelial cell adhesion molecules			
E-selectin	Mouse	R&D Systems	1:100
VCAM-1	Mouse	R&D Systems	1:1000
ICAM-1	Mouse	R&D Systems	1:1000
PECAM-1	Mouse	R&D Systems	1:1000
Growth factors			
EGF	Rabbit	Sigma	1:10
FGFa & b	Rabbit	Sigma	1:100
PDGF	Mouse	Sigma	1:10
TGFβ1,2,3	Mouse	Genzyme	1:1000
TGFβ1	Chicken	BBL	1:50
TGFβ2	Goat	BBL	1:50

Table 2.3 Secondary antibodies used for indirect immunofluorescence

Antibody raised against	Antibody raised in	Supplier	Dilution (In PBS)
Rabbit IgG (biotinylated)	Goat	Sigma	1:200
Mouse IgG (biotinylated)	Goat	Sigma	1:200
Chicken IgG (biotinylated)	Goat	Vector labs	1:200
FITC-streptavidin	-	Amersham	1:100

2.2 Studies of tissue homogenates

2.2.1 Source of biopsy material

A second set of normal skin biopsies was obtained from horses that were undergoing surgery or were being euthanised for other reasons as shown in Table 2.1. Four types of sarcoid were investigated; these were, verrucous, nodular, fibroblastic and mixed. Similar tissue was taken from horses with slow-healing granulating wounds with exuberant granulation tissue during normal surgical debridement prior to skin grafting. All samples were snap frozen in liquid nitrogen immediately and stored at -80°C until needed.

2.2.2 Biopsy dry weight

Each piece of frozen tissue was lyophilised in a freeze drier, xylene treated for 10 mins to remove excess fat, air dried and re-weighed on a Mettler, analytical balance. Biopsies were processed for either total protein, hydroxyproline, total collagen, type I procollagen (PICP) and TGFβ levels.

2.2.3 Protein extraction

Tissue samples were homogenised in 2M Nacl, 50mM phosphate buffer pH 7.4 to make a 5% homogenate. Protease inhibitors were added to the final concentrations indicated: 25mM ethylenediaminetetraacetic acid (EDTA), 25mM 6-aminohexanoic acid (EACA), 2mM phenylmethanesulphonyl fluoride (PMSF) and 10mM N-ethylmaleimide (NEM). Stock solutions of PMSF and NEM were first dissolved in ethanol. Aliquots were analysed for protein, total collagen, type I procollagen (PICP) and TGFβ levels.

2.2.4 Protein content of biopsies

The protein content was determined according to the method of Lowry et al, (1951), using folin phenol reagent (BDH). The optical density of the samples was measured at 750nm on a spectrophotometer (Shimadzu, uv-120-02). Bovine serum albumin (BSA) was used to construct a standard curve from which the protein content of the samples could be calculated. The total protein of the biopsies was expressed as μg/g dry weight.

2.2.5 Electrophoretic analysis of biopsy proteins

The biopsy samples were analysed by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), (Laemmli, 1970). Proteins were precipitated with an equal volume of ice cold 20% tetrachloroacetic acid (TCA). The samples were then washed in 5% TCA and extracted twice with ice cold acetone. After air drying, the proteins were denatured by heating at 100°C for 4 mins in 10µl Laemmli sample buffer (LSB) containing 0.0625M Tris/Hcl, 2% SDS, 0.002% bromophenol blue, 10% glycerol and reduced by breaking the disulphide bonds with 5% β-mercaptoethanol.

Electrophoresis was performed on slab gels consisting of a 7.5% separating gel (Appendix A) buffered at pH 8.8 and a 4% stacking gel (Appendix B) buffered at pH 6.8. Both separating and stacking gels were polymerised by the addition of the appropriate amounts of ammonium persulphate and N,N,N',N,' -tetramethyl-ethylene-diamine (TEMED). Other samples were electrophoresed on commercial 4-15% gradient gels (Biorad). Electrophoresis was carried out at 25mA/gel in an electrophoresis buffer containing 0.025M Tris/Hcl. 0.192M glycine and 0.1% SDS, pH 8.3, on a Biorad Minigel system. A duplicate set of samples was simultaneously run on a parallel gel. A negative control of Laemmeli sample buffer and high molecular weight standards were added to each gel (Novex- R&D Systems).

Immediately after electrophoresis the gels were stained with 0.1% Coomassie blue in methanol/acetic acid/water (4:1:5 v/v) for 30 mins. After excess stain was removed with destain (methanol/acetic acid/water 4:1:5 v/v). The gels was left to rehydrate in water overnight. After being photographed the gels were dried between two pieces of cellophane using a Hoffer "Easy Breeze" gel drier. A standard curve was

created by plotting relative mobilities (Rf value) against log molecular mass, where Rf value was calculated by measuring the distance migrated by the protein/marker and dividing by the distance migrated by the dye.

$$Rf = \frac{\text{distance migrated by protein}}{\text{distance migrated by dye}}$$

2.2.6 Collagen synthesis

The hydroxyproline method of Stegeman and Stadler (1967) was used to analyse the collagen content of the lyophilised tissue samples. Tissue samples were hydrolysed with 2ml 6M Hcl at 110°C overnight in test tubes sealed with marbles in a fume cupboard. The samples were evaporated in pyrex dishes over a boiling water bath to remove excess acid, re-evaporated with 2 ml distilled water and finally dissolved in 1 ml distilled water. Suitable aliquots were removed in duplicate from each sample and standards (0-20μg) made from a stock hydroxyproline solution (5mg trans-4-hydroxy-L-proline/50ml distilled water). The samples were made up to 2 ml with distilled water and 1ml 0.06M chloramine-T reagent (in 10ml distilled water, 10ml n-propanol, 80ml citric acid/sodium acetate buffer, pH 6.0) was added. After 20 mins at room temperature, 1 ml 15% aldehyde/perchloric acid solution (Ehrlichs or p-dimethyl-amino-benzaldehyde reagent) was added and the sample incubated at 60°C for 15 mins after vigorous shaking. The optical density of the product was measured at 550 nm on a spectrophotometer and the hydroxyproline content calculated from a hydroxyproline standard curve. Collagen content of the tissue samples was calculated by assuming that it contains 13.6% hydroxyproline (Shah et al., 1994) and expressed as μg/g of the total biopsy dry weight.

2.2.7 PICP procollagen determination

The PICP assay (Orion Diagnostica, Finland) was based on the standard radioimmunoassay. A sample containing an unknown amount of type I procollagen (PICP) to be assayed was mixed with a standard amount of a radioactively labelled derivative of type I (PICP). The labelled and unlabelled antigens were then allowed to compete for the limited number of high affinity binding sites of the antibody. The amount of radioactive antigen in the antigen-antibody complex was inversely proportional to the amount of unlabelled antigen in the reaction mixture. After washing away the free antigen, the residual radioactivity was counted and the actual concentration was calculated with the aid of a standard curve based on known amounts of unlabelled antigen analysed in parallel with the unknown.

An aliquot of tissue homogenate (100µl) was mixed with 200µl of PICP antiserum and 200µl ^{125}I-labelled PICP. After a 2 hour incubation at 37°C, separation reagent is added, tubes are allowed to stand for 30 min at room temperature and then centrifuged for 15 mins at 2000 x g at 4°C. The supernatant was then removed and the sediment containing the precipitated antibody-antigen complex was counted in a, Rackgamma II (LKB Wallac.) gamma counter for 1min/tube.

A standard curve was produced by calculating the binding of five standards as a percentage of the maximum possible binding and plotting these values. The CPM (counts per min) results for unknowns were then expressed in terms of bound radioactivity. PICP values were read directly from the standard curve.

2.2.8 TGFβ analysis

The quantities of TGFβ in the tissue homogenate were measured using an ELISA

method (Genzyme). In order to measure biologically active TGFβ, it was necessary to activate the samples, standards and controls. This was done by diluting and acidifying them with HCl for 1 hour at 4°C. Acidified samples and standards were neutralized to pH 7.0-7.4 with NaOH when they could be aliquoted and frozen at -20°C until required for assay.

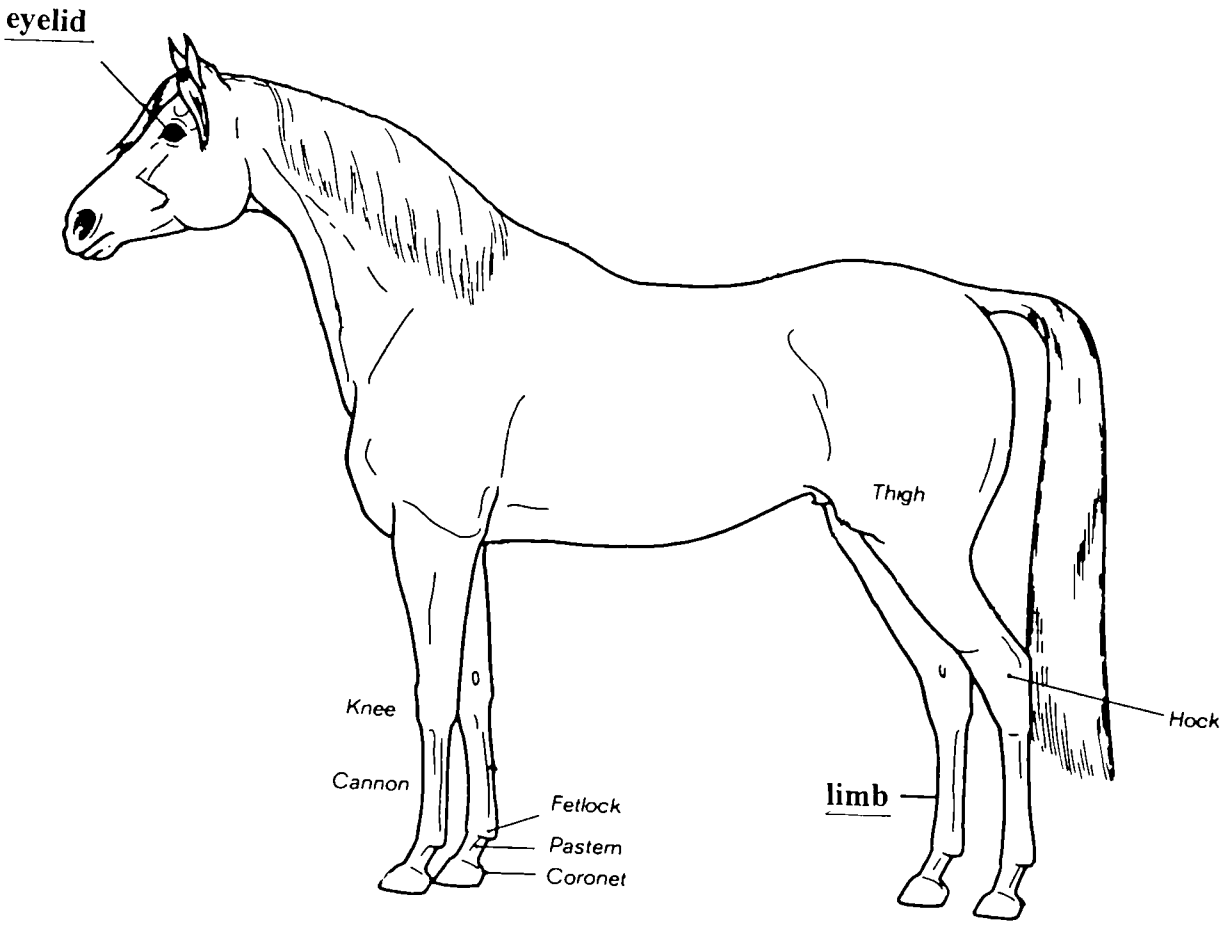
A measured volume (100μl) of activated sample, standard or control was added to each test well in duplicate and incubated at 37°C for 60 mins to allow any TGFβ present to be bound by antibodies on the microtiter plate. The wells were then washed and 100μl of direct-labelled HRP-conjugated polyclonal antibody to TGFβ was added which binds to the captured TGFβ during incubation. After washing, 100μl of a substrate solution (tetramethylbenzidine) was added to the wells, and the plates were incubated at room temperature for 20 minutes, producing a blue colour in the presence of peroxidase. The colour reaction was then stopped by the addition of acid which changed the blue colour to yellow. The intensity of the yellow colour is directly proportional to the amount of TGFβ present in the sample, standard or control. The absorbance of each well was read at 450nm and a standard curve was constructed to quantitate TGFβ concentrations in the controls and samples.

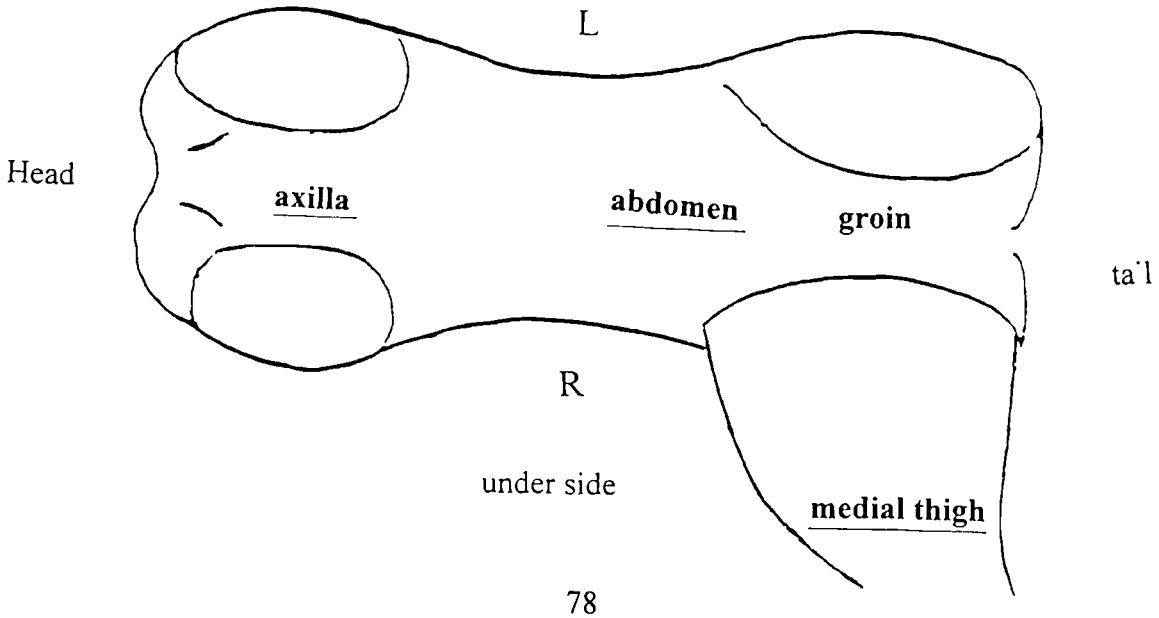
2.3 Cell culture studies

2.3.1. Source, explantation and culture of equine dermal fibroblasts

Dermal Tissue was obtained post mortem from horses and a pony which had been killed for other reasons. The source of the tissue used for cell culture experiments is shown in Table 2.4. Six different anatomical sites were sampled (Figure 2.1). These

Figure 2.1 Diagrammatic representation of the six sites of the horse used in cell culture studies





were: eyelid, axilla, groin, medial thigh, ventral midline and limb (fore, mid-dorsal cannon). Similar tissue was taken from horses with slow-healing granulating wounds with exuberant granulation tissue during normal surgical debridement prior to skin grafting. One of these horses (case 10026) had a vertical wound which was healing normally above the knee and a non-healing exuberant granulating wound below the knee (Plate 2.1).

Table 2.4 Source of explantation tissue used in cell culture experiments

TYPE OF TISSUE	BREED	SEX	AGE
Normal	Hackney	Gelding	13
	TB	Gelding	5
	Bay Roan	Gelding	9
	TB	Gelding	10
Granulation	Arab	Mare	7
	TB	Male	1
	Arab	Filly	4
	TB	Gelding	6
Sarcoids	TB	Mare	8
	Welsh	Mare	14
	TB	Gelding	7
	Hunter	Gelding	12

Samples were taken from superficial, middle and deep sites within the exuberant granulation tissue.

Tissue samples were taken from both the healing and non-healing areas of the wound of the horse in Plate 2.1. The age of the granulation tissue ranged from 3-8 weeks

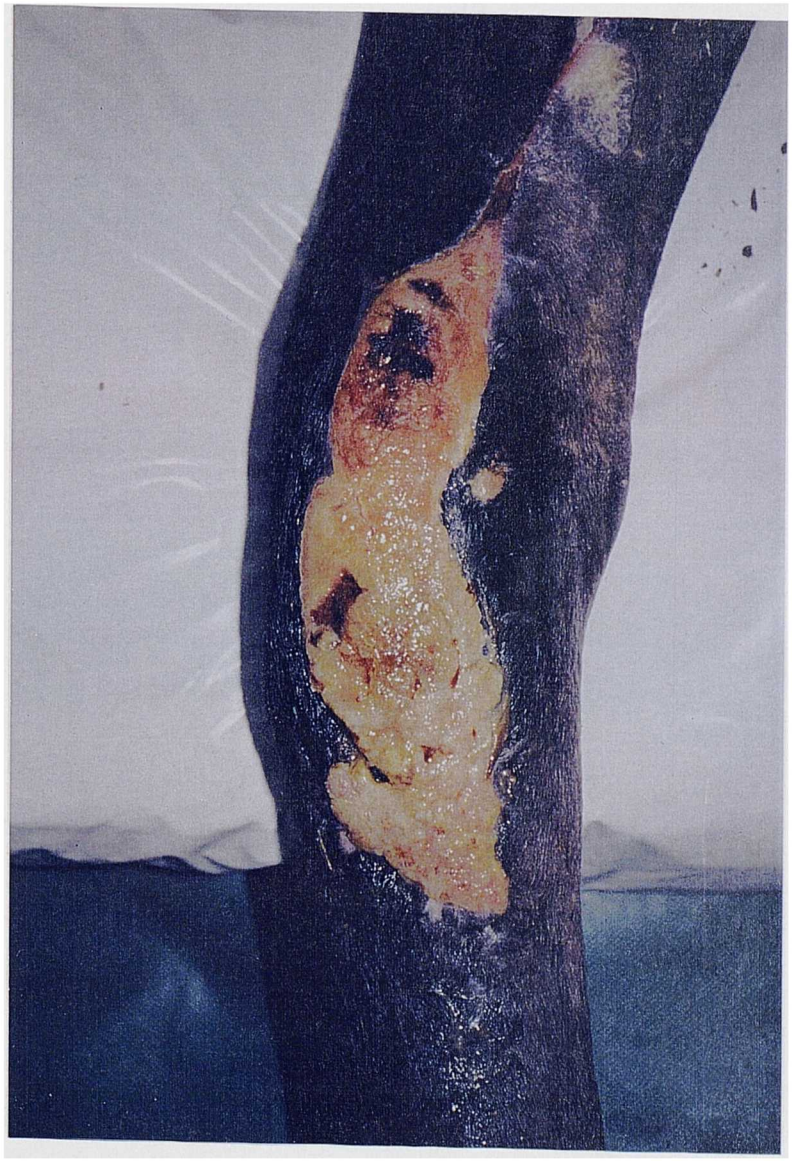


Plate 2.1 Case 10026. Spontaneously the wound healed well above the knee, less well at the knee itself and failed to heal below the knee

old. Horses with sarcoids which were being euthanised also had dermal tissue removed. Four types of sarcoid were investigated as previously described (section 2.1.1.). Tissue samples were immediately transferred to a dish of Hank's balanced salt solution (Gibco) and washed. Samples were cut into 3-5 mm^2 pieces and placed into 25 cm^2 tissue culture flasks (Nunc, Gibco) containing DMEM (Gibco), supplemented with 10% Foetal calf serum (Sigma), 20 mM Hepes buffer, 100 μg/ml gentamicin and 0.5 μg/ml amphotericin B (Gibco) in a 5% carbon dioxide in air, 37°C environment.

Readiness for subculturing was determined by the extent of fibroblast cell outgrowth (5-10 days). Cells were farmed successively in a 1:4 split ratio to passage 3-8 for experimental use.

2.3.2 Source, explantation and culture of equine epithelial cells

Equine skin was obtained post mortem from horses which had been killed for other reasons (Table 2.4). Six different anatomical sites were sampled, as described in section 2.3.1. Tissue samples were immediately transferred to a dish of Hank's balanced salt solution (Gibco) and washed. The dermal part of the tissue was removed using a scalpel. The samples were cut into 3-5 mm^2 pieces and treated as described in section 2.3.1. and placed into 25 cm^2 tissue culture flasks (Nunc, Gibco) containing DMEM (Gibco), supplemented with 10% Foetal calf serum (Sigma), 20 mM Hepes buffer, 100 μg/ml gentamicin and 0.5 μg/ml amphotericin B (Gibco), 50 ng/ml insulin and 50 ng/ml hydrocortisone (Sigma) in a 5% carbon dioxide in air, 37°C environment.

Readiness for subculturing was determined by the extent of epithelial cell outgrowth (5-10 days). Pure cultures were obtained by eliminating fibroblast cells by

selective trypsinisation using 0.5% trypsin/EDTA (Gibco) for 5 minutes at 37°C. The trypsinisation was stopped by the addition of fresh medium. The established cultures were able to stratify and form differentiating multi layers and the cells were subcultured for many passages.

2.3.3 Anti-cytokeratin staining

The epithelial cells were plated onto 35mm dishes (Gibco). After confluence had been reached, the media was removed and the cell layer washed with PBS. Cells were then fixed in methanol at -20°C for 10 minutes. After further washing with PBS the cells were permeabilised with 0.2% triton X-100 in PBS for 5 mins at room temperature. The cells were rewashed as before and blocked with endogenous peroxidase (1 part 3% hydrogen peroxide to 9 parts methanol) for 30 mins. After rinsing the cells goat serum (10% in PBS) was added to block non-specific binding of the antibody. The cells were rewashed and the primary antibody (α-cytokeratin, Sigma) was added at 1:1000 in PBS, overnight at 4° C. After the primary incubation the cells were washed in PBS-tween 20. The second antibody, biotinylated goat anti-mouse IgG, (Sigma) was added at 1:2000 in PBS with 1% BSA at room temperature for 1 hour. The cells were again washed in PBS/tween 20 and extra-avidin peroxidase (Sigma) was added at 1:500 for 20 mins at room temperature. After the final washings, the enzyme substrate was added, DAB (Sigma) in nickel chloride at room temperature for 5 mins and the reaction was stopped with PBS or distilled water.

2.3.4 Cell proliferation

Normal fibroblasts cells from all of the six normal skin sites, cells derived from

exuberant granulation tissue and those from verrucous, nodular, fibroblastic and mixed sarcoid tumours were harvested from stock dishes. Single-cell suspensions of these cells were plated onto 35 mm plastic culture dishes (Nunc) in duplicate at $6x10^4$ cells/dish in 1ml DMEM supplemented with 2.5% foetal calf serum and 1% penicillin-streptomycin, 20 mM Hepes buffer (Gibco). Cells were left to attach for six hours, after which the plating medium was replaced with "experimental" medium: DMEM, 1% penicillin-streptomycin, 20 mM Hepes buffer, and either 2.5 or 0% foetal calf serum. Medium was changed every two days. Cells from duplicate dishes were released with 0.5% trypsin/EDTA solution (Gibco), 5 mins at 37°C and counted in a Neubauer counting chamber, every five days up to and including day twenty. All of the results were expressed as the mean ± standard error (SEM). The statistical significance of the results was assessed using Student's t-test and a p value of <0.05 was considered to be significant.

2.3.5 Morphological examination of cells

Photomicrographs were taken 48 hours after subculturing using an Olympus CK2 inverted microscope with a photomicrograph attachment PM10-AK-2 and an Olympus C35 DA/2 camera. Kodak Ektachrome 64T tungsten film was used from which prints were made.

2.4 Response to exogenous growth factors in culture

2.4.1 Source, explantation and culture of dermal fibroblasts

Dermal Tissue used in these studies is shown in Table 2.4. The tissue samples

were cultured as described in section 2.3.1.

2.4.2 Cell proliferation

Normal fibroblasts cells from all of the six sites, cells derived from exuberant granulation tissue and those from verrucous, nodular, fibroblastic and mixed sarcoid tumours were harvested from stock dishes. Single-cell suspensions of cultured cells from normal skin, granulation tissue and sarcoid lesions was plated onto 35 mm plastic culture dishes (Nunc) in duplicate at $6x10^4$ cells/dish in 1ml DMEM supplemented with 2.5% foetal calf serum and 1% penicillin-streptomycin, 20 mM Hepes buffer (Gibco). Cells were left to attach for six hours, after which the plating medium was replaced with "experimental" medium: DMEM, 1% penicillin-streptomycin, 20 mM Hepes buffer, 2.5% foetal calf serum containing either 10 ng/ml EGF (Sigma), 10 ng/ml FGFa and FGFb or 1ng/ml TGFβ (R&D Systems). Medium was changed every two days. Cells from duplicate dishes were released with 0.5% trypsin/EDTA solution (Gibco), 5 mins at 37°C and counted in a Neubauer counting chamber, every five days up to and including day twenty. All of the results were expressed as the mean ± standard error (SEM). The statistical significance of the results was assessed using Student's t-test and a p value of <0.05 was considered to be significant.

2.4.3 Photomicroscopy

Photomicrographs were taken (section 2.3.5.) 48 hours after subculturing using an Olympus CK2 inverted microscope with a photomicrograph attachment PM10-AK-2 and an Olympus C35 DA/2 camera. Kodak Ektachrome 64T tungsten film was used from which prints were made.

2.5 Synthesis of extracellular matrix molecules by dermal fibroblasts

2.5.1 Source, explantation and culture of dermal fibroblasts

Dermal fibroblasts were cultured from tissue described in Table 2.4. Media samples were collected from early passage cells, aliquoted and stored at -20°C for PICP and TGFβ analysis.

2.5.2 Collection of plasma and wound fluids

Peripheral blood (10 ml) was collected from the horses in lithium/heparin tubes, mixed well and centrifuged for 15 min, 3,900 g at 4°C. The plasma was carefully removed to avoid disturbing the red cells in the bottom of the tube. Plasma samples were aliquoted and stored at -20°C until required for analysis.

Wound fluid was collected from dressings that had been applied to non-healing wounds. The dressings were changed daily and the wound fluids were extracted. The wound fluids were centrifuged at 4°C for 15 mins at 1,500 r.p.m. Wound fluids were then aliquoted and stored at -20°C until required for biochemical analysis.

2.5.3 Immunocytochemical analysis of extracellular matrix deposition cell adhesion molecules and growth factors

Dermal fibroblasts were plated at a density of 2 x 10^4 cells/well on eight well culture slides (Gibco) in standard medium. Following cell attachment the plating

medium was replaced with experimental medium: DMEM containing 40μg/ml ascorbic acid and supplemented with 2.5% FCS. The cells were then cultured for a further 72 hours.

Cultures were washed in phosphate buffered saline (PBS), fixed in 4% paraformaldehyde in PBS for 5 mins and rewashed with PBS. The cells were then permeabilised with 0.1% Triton X-100 in PBS for 5 mins and rewashed in PBS. The cell cultures were stained immunocytochemically with monoclonal antibodies against extracellular matrix molecules, growth factors and cell adhesion molecules (Table 2.2). Incubations with primary antibodies were for 1 hour, followed by 3 x 5 min washes with PBS. This was followed by incubations with the appropriate biotinylated secondary antibodies (Table 2.3). After further washing the cells were incubated with FITC-conjugated streptavidin for 20 minutes at room temperature. The cultures were washed again in PBS and mounted in vectashield (Vector laboratories) with coverslips. The stained sections were visualised by epifluorescent microscopy using a Leitz Orthoplan microscope fitted with a Leitz Orthomat automatic camera and photographed using Kodak Ektachrome 400 film.

2.5.4 PICP procollagen determination

The PICP assay (Orion Diagnostica, Finland) was based on the standard radioimmunoassay. The method was described in section 2.2.7.

2.5.5 TGFβ analysis

The quantities of TGFβ in the conditioned medium was measured using an ELISA method (Genzyme), as described in section 2.2.8

2.5.6 Lowry protein and hydroxyproline estimations

The quantities of protein and hydroxyproline were measured in serum and wound fluids from the horses described above. The methods used are described in sections 2.2.4 and 2.2.6 respectively.

2.5.7 Metabolic labelling of cell cultures

The ability of normal dermal fibroblasts, granulation tissue and sarcoid derived fibroblasts to synthesise collagen and other proteins was investigated.

Normal, granulation tissue and sarcoid derived fibroblasts were plated out in triplicate at 1 $x10^5$ cells/35mm dish in standard medium containing 2.5% FCS. Cells were incubated for an initial period of 24 hours at 37°C in a humid environment containing 5% CO_2 in air. The cells were labelled with 20μCi/ml L-[5-^{3}H]-proline in DMEM with 50μg/ml ascorbic acid and 2.5% FCS for collagen analysis. Identical incubation volumes were maintained throughout the experiment to ensure an equal amount of radiolabel was added to each culture and the cells were incubated for a further 24 hours.

The cell numbers in duplicate parallel cultures were determined before and after treatment using a Neubauer counting chamber. The conditioned medium from these dishes was kept at -20°C for further analysis.

2.5.8 Preparation of media and cell layer fractions

The conditioned media was removed from the cultures and the cell layer was washed twice with 1 ml ice cold PBS. The washes and the media were pooled and protease inhibitors added (2mM PMSF, 10mM NEM, 25mM EACA, 25mM EDTA). Proteins in the cell layer were extracted with 1 ml 4M guanidium hydrochloride in 50mM Tris/HCl buffer, pH7.4 containing protease inhibitors (0.5mM PMSF, 2mM NEM, 2mM EACA, 2mM EDTA) at 4°C for 24 hours. Solubilised proteins were removed and the dish was scraped and rewashed with 1ml PBS. The proteins and washes were pooled and collected in preweighed bijou bottles and reweighed after preparation. All samples were stored at -20° C until analysis. Media and cell layer fractions were analysed separately and in triplicate for total protein and collagen.

2.5.9 Analysis of synthesised protein

Protein synthesis was determined by the incorporation of [5-^{3}H]-proline into TCA precipitable counts. Protein aliquots or media or cell extract were precipitated using ice-cold 20% TCA for 1 hour at 4°C, centrifuged for 10 min at (10,000g) at 4°C. Proteins were then washed with 5% TCA and extracted twice with ice-cold acetone. Samples were air dried and solubilised in 1M NaOH by boiling for 3 min. After neutralisation with 1M HCl and the addition of scintillation fluid, the samples were counted in a liquid scintillation counter. The results were expressed as disintegrations per minute (dpm) per 10,000 cells which was calculated from the cell numbers at the end of the experiment.

2.5.10 Analysis of synthesised collagen

Collagen was assayed by a modification of the procedure described by Peterkovsky & Diegelmann (1971). Briefly, 1 ml media and cell extract samples labelled with [5-^{3}H]-proline were dialysed against collagenase buffer containing 50mM Tris/HCl, 5mM calcium chloride, 5mM NEM, 1mM PMSF, pH 7.4 at 4°C. Three 100µl aliquots of cell supernatants and cell extracts were digested with highly purified bacterial collagenase (1.25 units in 10µl in 10mM calcium acetate, 25mM Tris/HCl, pH 7.2) and three aliquots were left untreated as controls. Samples were incubated at 37°C for 4 hours and the digestion was stopped by precipitation with ice-cold 20% TCA. Samples were washed with 5% TCA, extracted twice with acetone and assayed for ^{3}H-proline content in non-collagenous and total protein as before. To correct for the enriched proline content of collagen, the percentage collagen was determined as follows: (dpm collagenous protein x 100)/([5.4 x dpm non-collagenous protein] + dpm collagenous protein) (Peterkovsky, 1972).

2.6 Zymography

2.6.1 Source of tissues and culture of dermal fibroblasts

Dermal tissue was obtained aseptically from the horses previously described in Table 2.4. Tissue samples were immediately transferred to a dish of Hank's balanced salt solution (Gibco) and washed. Samples were cut into 3-5 mm^2 pieces and placed into 25 cm^2 tissue culture flasks (Nunc, Gibco) containing DMEM (Gibco), supplemented with 10% Foetal calf serum (Sigma), 20 mM Hepes buffer, 100 µg/ml gentamicin and 0.5 µg/ml amphotericin B (Gibco) in a 5% carbon dioxide in air, 37°C environment.

Readiness for subculturing was determined by the extent of fibroblast cell

outgrowth (5-10 days). Cells were farmed successively in a 1:4 split ratio to passage 3-8 for experimental use. Media samples were collected from early passage cells, with and without treatment with exogenous growth factors as previously described (section 2.2.4), aliquoted and stored at -20°C until required for analysis.

2.6.2 Collection of plasma and wound fluids

Normal peripheral blood was collected from the horses mentioned in section 2.5.2. Plasma samples were separated from the red blood cells (Section 2.5.2) aliquoted and stored at -20°C until required for analysis.

Wound fluid was collected from dressings that had been applied to non-healing wounds. The dressings were changed daily and the wound fluids were extracted as described in (section 2.5.2). Wound fluids were then aliquoted and stored at -20°C until required for analysis.

2.6.3 Tissue samples

Tissue samples were collected and homogenised from the same horses as described in section 2.2.1.

2.6.4 Zymography

Metalloproteinases in serum, wound fluids, tissue homogenates and cell supernatants were analysed by substrate-gel zymography using sodium dodecylfulfate (SDS)-polyacrylamide gel electrophoresis, described previously (Laemmli, 1970).

2.6.5 Treatment of samples

Briefly, samples were incubated at 37°C for 1 hour at the appropriate dilution. The samples were diluted with non-reducing sample buffer (1M Tris/HCl pH6.8, 0.5% SDS (w/v), 1.7% glycerol (v/v), 0.05% (w/v) bromophenol blue) and distilled water. The incubation allows the SDS to breakdown the Van der Waals forces and hydrophobic interactions within the structure of the enzymes and so renders them inactive. The SDS also gives the enzymes a net negative charge, so that they are separated by size and not charge.

2.6.6 Preparation of resolving gel

A Miniprotean II gel electrophoresis system (Biorad, Hemel Hempstead, Herts) was used in the zymography experiments. Gloves were worn to prevent the introduction of proteases from the skin into the samples. Prior to use, all of the equipment was cleaned using ethanol, to remove any proteases which may be present.

A 7.5% gelatin resolving gel was made (appendix C) by copolymerising acrylamide/bisacrylamide (Severn Biotech Ltd., Kidderminster) stock, with gelatin. This produced an 0.25% gelatin/7.5% acrylamide gel. The gel was cast using 3.2 ml of the gel solution into each of the sets of glass plates. Once the gel was set, (30 - 45 min), the stacking gel was added.

2.6.7 Preparation of stacking gel

A 4% stacking gel buffered at pH 6.8 was used (Appendix B). This gel was used as the pore size is large and therefore no molecular sieving occurs at this stage to ensure

that all of the samples enter the resolving gel at the same time point, thus allowing molecular weights to be calculated more accurately. Wells were made in the gel by placing a comb between the glass plates immediately after the stacking gel had been cast. The gel was then left to set, which took about 30 min.

2.6.8 Loading of samples and running of gels

Once the stacking gel was set the comb was carefully removed and the wells were filled with 1x running buffer (10x running buffer: Tris 15g/L, Glycine 72g/L and SDS 5g/L in distilled water pH 8.3) and the samples were loaded. Either 5µl or 10µl was added to the wells depending on whether a 10 or 15 well comb was used. The plates were then fitted to the gel assembly unit and this was then filled with 1x running buffer. Gels were electrophoresed at a constant 200V for 45 - 60 min or until the dye front reached the bottom of the resolving gel. A set of high molecular weight markers were run on each gel (Novex) as described in section 2.2.5.

2.6.9 Treatment of markers

When the gel run was completed, the stacking gel was removed and the resolving gel was marked to provide the correct orientation. The marker lane was removed and placed in Coomassie blue stain (0.5%; 40% MeOH, 10% acetic acid) for 30 min. The marker lanes were then placed in destain (40% MeOH, 10% acetic acid) overnight or until the markers became clearly visible. Markers were then washed in water prior to drying.

2.6.10 Treatment of gels

The resolving gels were washed in 2.5% (v/v) Trition X-100 for 1 hour, to remove any SDS and then repeatedly rinsed in distilled water to remove the Triton X-100. The resolving gels were placed in 1x gelatin refolding buffer (Appendix D) and incubated for 24 hours at 37°C. The gels were incubated in refolding buffer to allow the enzymes to reform their secondary structure and become active.

Following the incubation the gels were placed in 2% Coomassie blue stain for about 30 min and destained for about 45 -60 min, until the digestion bands became clear. The latent (zymogens) and active forms of the enzymes were visualised, as the enzymes underwent autolytic cleavage of the cysteine switch during the refolding process and so the molecular weight of the active and latent form could be established.

The gels were washed in distilled water for 1 hour, and dried as described in section 2.2.5. A standard curve was created and the Rf values were calculated as previously described (section 2.2.5).

CHAPTER THREE

The histology, extracellular matrix and growth factor distribution in equine chronic wound healing

3.1 INTRODUCTION

During wound healing there is an initial influx of inflammatory cells into the wound site that is regulated by the expression of endothelial cell adhesion molecules (Pober & Cotran, 1991). The activation of these inflammatory cells and platelet degranulation, allows the release of a "cocktail" of cytokines including growth factors which stimulate the inward migration of fibroblasts and endothelial cells. Fibroblasts are known to be the major producers of extracellular matrix molecules during wound healing. They form the basis of granulation tissue which is composed of fibronectin and collagen type III and subsequently a more mature dermis of collagen type I. This is remodelled into scar tissue (Kurkinsen *et al.*, 1980).

This normal progression of wound healing involving inflammation, granulation tissue formation and remodelling is disrupted in chronic wounds.

Chronic equine wounds have been characterised by the formation of exuberant granulation tissue (Bertone *et al.*, 1985; Bertone, 1988; Blackford, *et al.*, 1986; Fretz *et al.*, 1983; Jacobs *et al.*, 1984; Lee & Swain, 1988; Lindsay, 1988), which prevents normal cellular/extracellular matrix interactions. These support epidermal migration and enable wound closure to take place.

There are no published studies documenting the histopathological, cytological

features, or tissue architecture of chronic equine wounds. Such investigations may lead to new knowledge of the pathogenesis of chronic equine wound healing and perhaps suggest novel treatment regimes. The aim of this study was to document the appearance of the tissue architecture, extracellular matrix molecules, endothelial cell adhesion molecules and growth factors during equine wound healing.

3.2 METHODS

3.2.1 Source of tissue

Skin biopsies were taken from horses under general anaesthesia as described in Chapter 2, section 2.2.1.

3.2.2 Immediate treatment of biopsy

The tissue samples were either fixed in 4% paraformaldehyde or placed in OCT embedding compound as described in chapter 2, section 2.2.2.

3.2.3 Histological examination

After fixation the specimens were dehydrated through a series of alcohols, cleared and embedded in molten wax and later, sectioned and stained as described in Chapter 2, section 2.2.3.

3.2.4 Immunocytochemical examination

Chapter 2, section 2.2.4. describes how the frozen blocks were sectioned and stained with primary and secondary antibodies (see Table 2.2 and 2.3 respectively).

3.3 RESULTS

3.3.1 Histological features of healing equine wounds

On histological examination the wound area and the surrounding skin could be identified. The surrounding skin appeared normal (Plate 3.1a). Collagen fibres stained green, a thin layer of fine collagen fibres can be seen in the papillary dermis. However thicker collagen fibres were observed in the subpapillary dermis especially around newly forming hair shafts in the wound site. The wound space was filled a with thicker

diameter collagen fibre meshwork arranged roughly parallel to the epidermal surface (Plate 3.1b). Plate 3.2 demonstrates the architecture of granulation tissue in the wound space. Large numbers of cells, fibroblasts and inflammatory cells were evident within the tissue and newly formed, thin-walled blood vessels were clearly visible.

3.3.2 Extracellular matrix molecule distribution in equine wounds

A) Laminin

Laminin was localised in the epidermal and endothelial cell basement membranes (Plate 3.3a). Within the wound area laminin was present in the basement membranes of the blood vessels (Plate 3.3b).

B) Fibronectin

Fibronectin staining was most prominent in epidermal basement membranes at the dermal-dermal junctions (Plate 3.4a). The most intense staining was adjacent to the basement membrane of the epidermis and hair follicles. Enhanced staining was seen around blood vessels, especially in the papillary dermis (Plate 3.4b), with a particularly noticeable broad cuff of staining around the blood vessel. A more diffuse staining was evident at the wound edge (Plate 3.4c). A similar pattern of staining was seen when sections were stained with fibrin (Plate 3.5).

C) Collagen types I and IV

Collagen staining was widespread through the extracellular matrix of the tissue sections. In the upper dermis collagen I appeared in a net-like structure (Plate 3.6a), but in the lower dermis appeared to have a more fibrillar structure (Plate 3.6b). Collagen I was present around some blood vessels (Plate 3.6c) in the lower dermis, displaying a similar cuffed appearance to laminin and fibronectin.

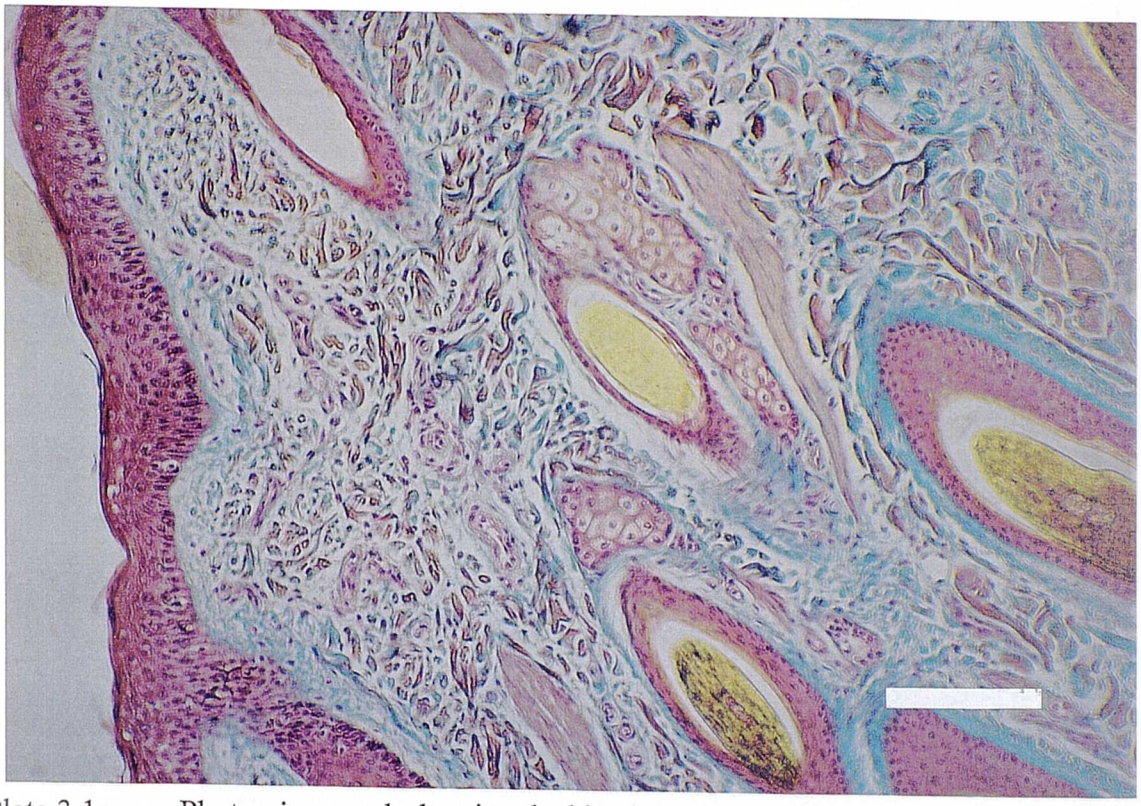


Plate 3.1a Photomicrograph showing the histology of the skin surrounding a wound. Section stained with Masson's Trichrome. Collagen is stained green. Bar: 100μm

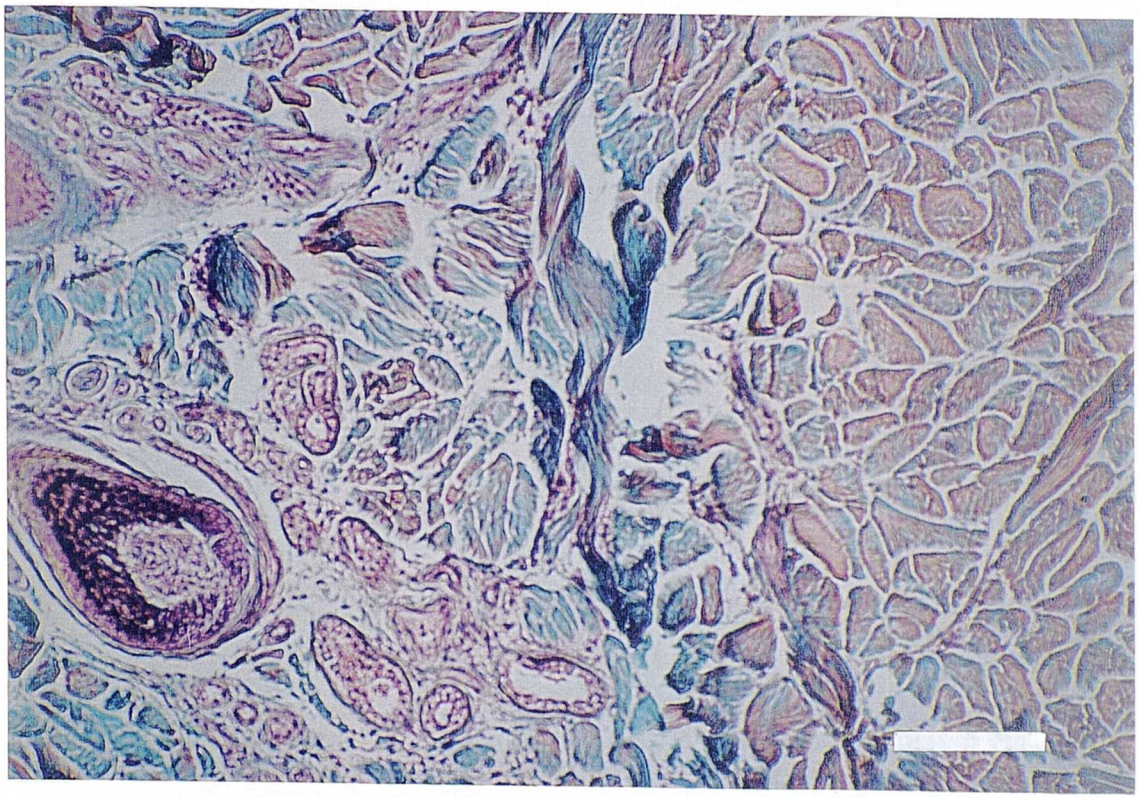


Plate 3.1b Photomicrograph showing the histology of the wound space with a thicker diameter collagen fibre network. Section stained with Massons Trichrome. Bar: 100μm

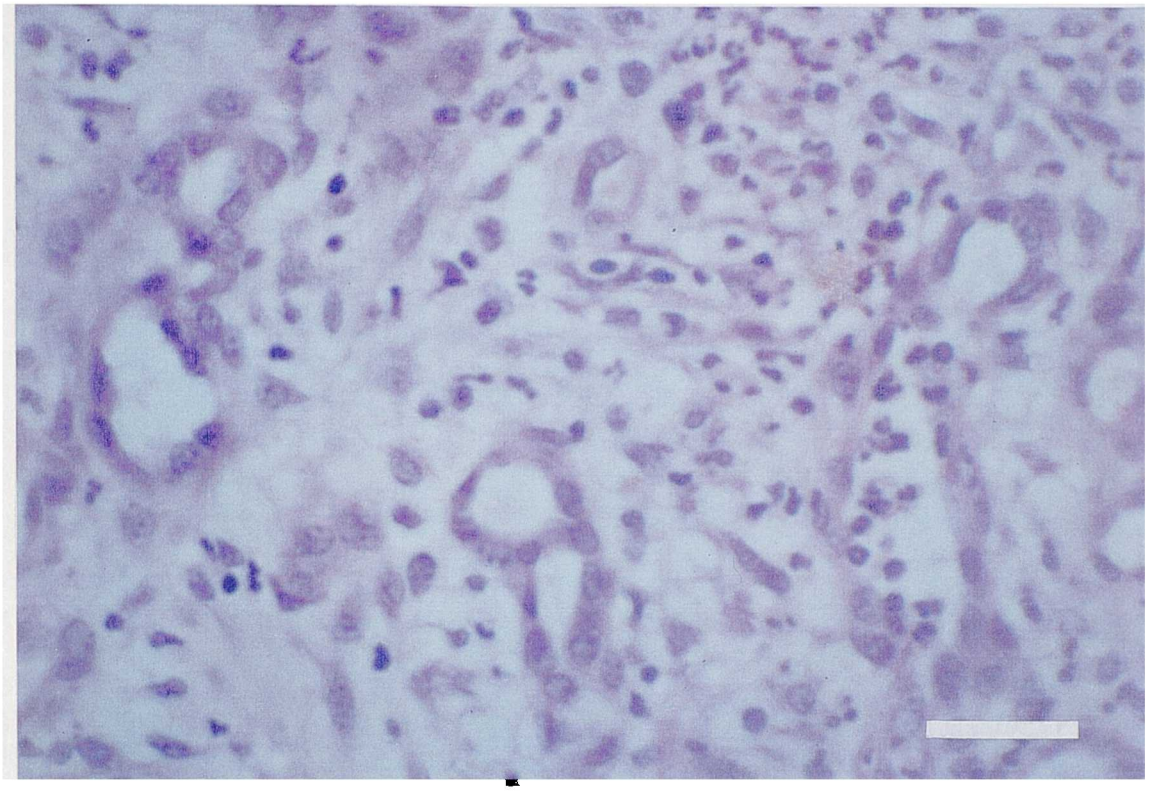


Plate 3.2 Photomicrograph of the histology of exuberant granulation tissue in the wound. Stained with H & E. Bar: 100μm

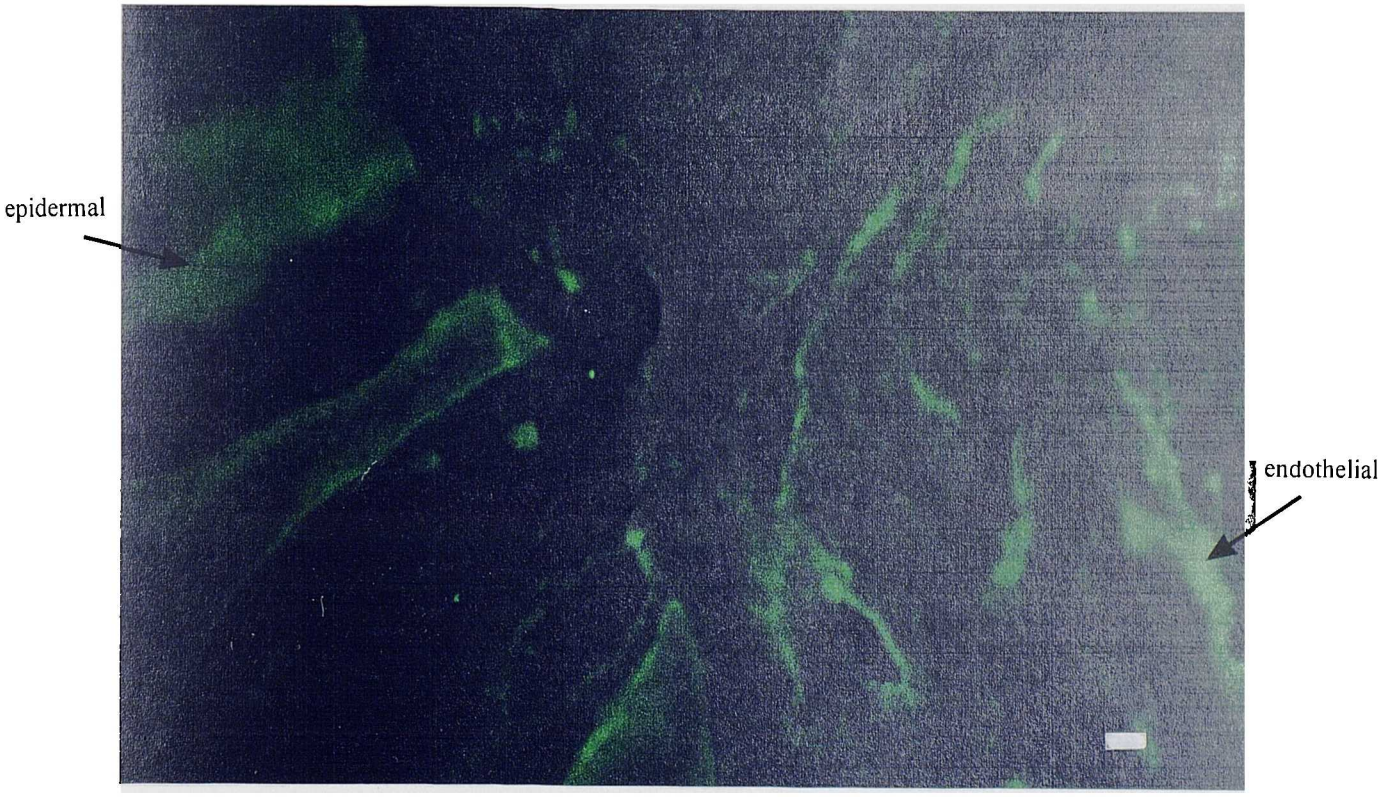


Plate 3.3a Photograph of laminin localisation in the epidermal and endothelial cell basement membranes. Bar:100 μm

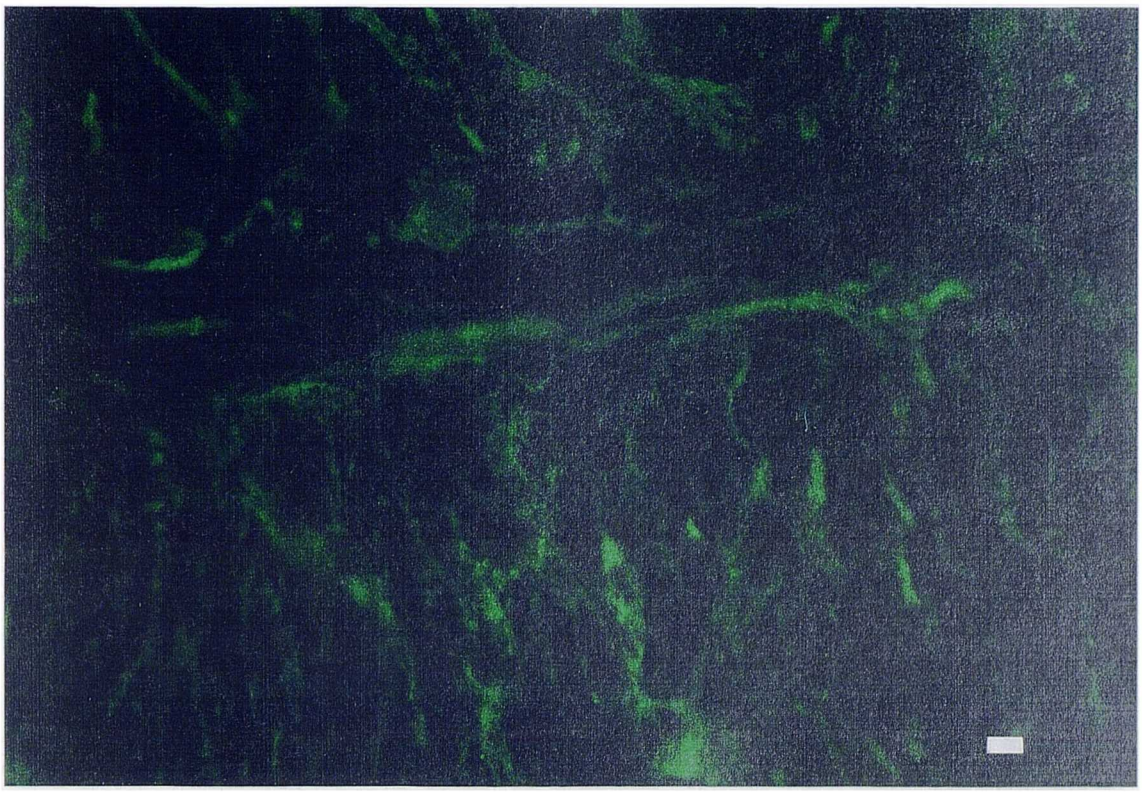


Plate 3.3b Photomicrograph showing the presence of laminin in the basement membrane of blood vessels. Bar: 100μm

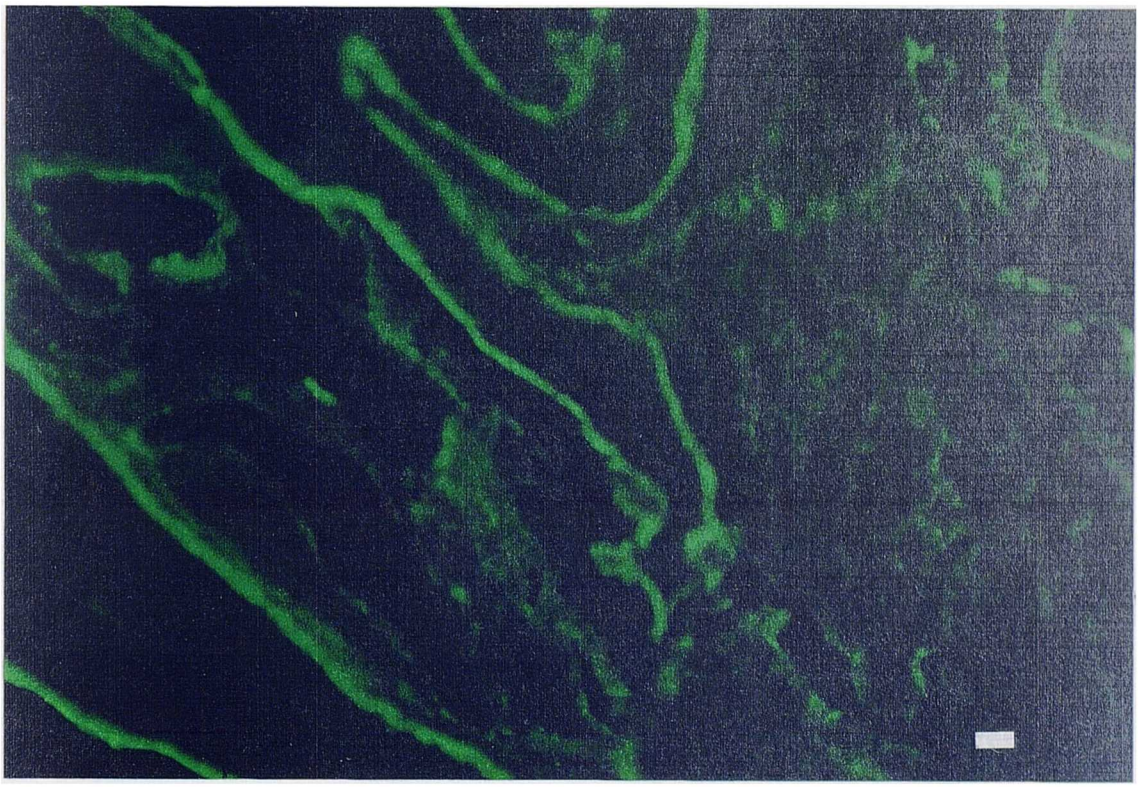


Plate 3.4a Photomicrograph showing fibronectin immunolocalisation in the epidermal basement membranes. Bar: 100μm

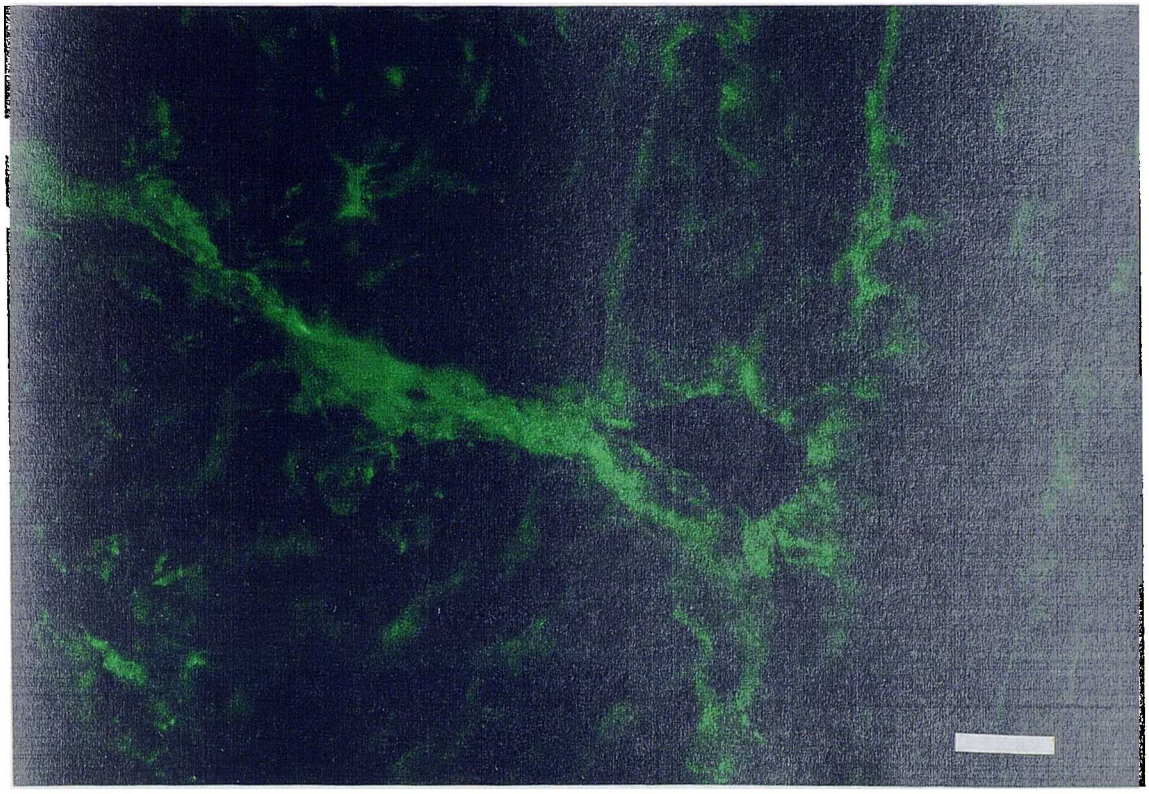


Plate 3.4b Photomicrograph showing enhanced staining for fibronectin around blood vessels in the papillary dermis. Bar: 100μm

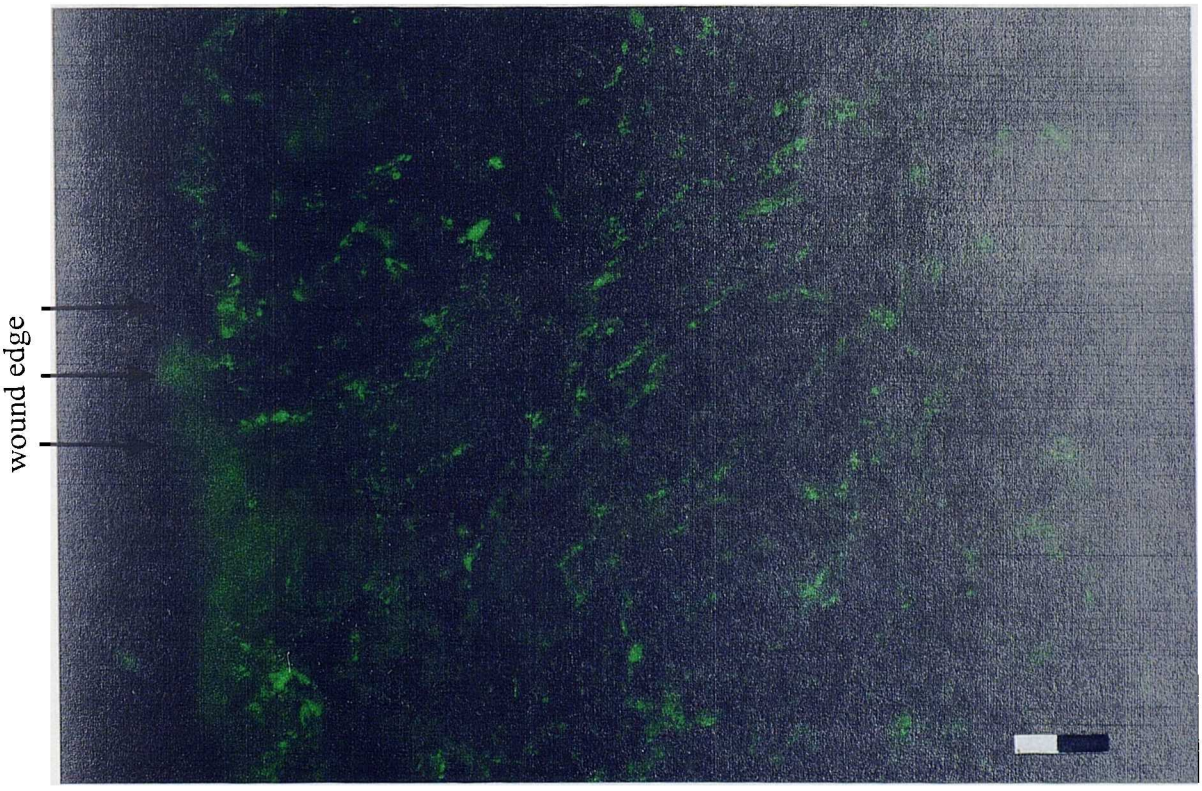


Plate 3.4c Photomicrograph showing diffuse fibronectin staining at the wound edge. Bar: 100 μm Limited fibronectin was found in the wound bed.

Type IV collagen staining localised in the epithelial and endothelial basement membranes (Plate 3.7a). Sweat and sebaceous glands stained intensely for type IV collagen (Plate 3.7b). Blood vessels in the deep dermis stained strongly with type IV collagen, displaying the cuffed appearance as with collagen I, laminin, fibronectin and fibrin (Plate 3.7c).

3.3.3. Distribution of cell adhesion molecules in equine wounds

A consistent pattern and intensity of staining was seen for all of the endothelial cell adhesion molecules studied. There appeared to be no alteration in the distribution of these adhesion molecules in fresh or older wounds.

A) E-selectin (Endothelial leucocyte adhesion molecule-1)

E-selectin staining was sparse in the skin sections (Plate 3.8a). More intense staining was observed around blood vessels in the wound area (Plate 3.8b). This adhesion molecule was only expressed by blood vessels.

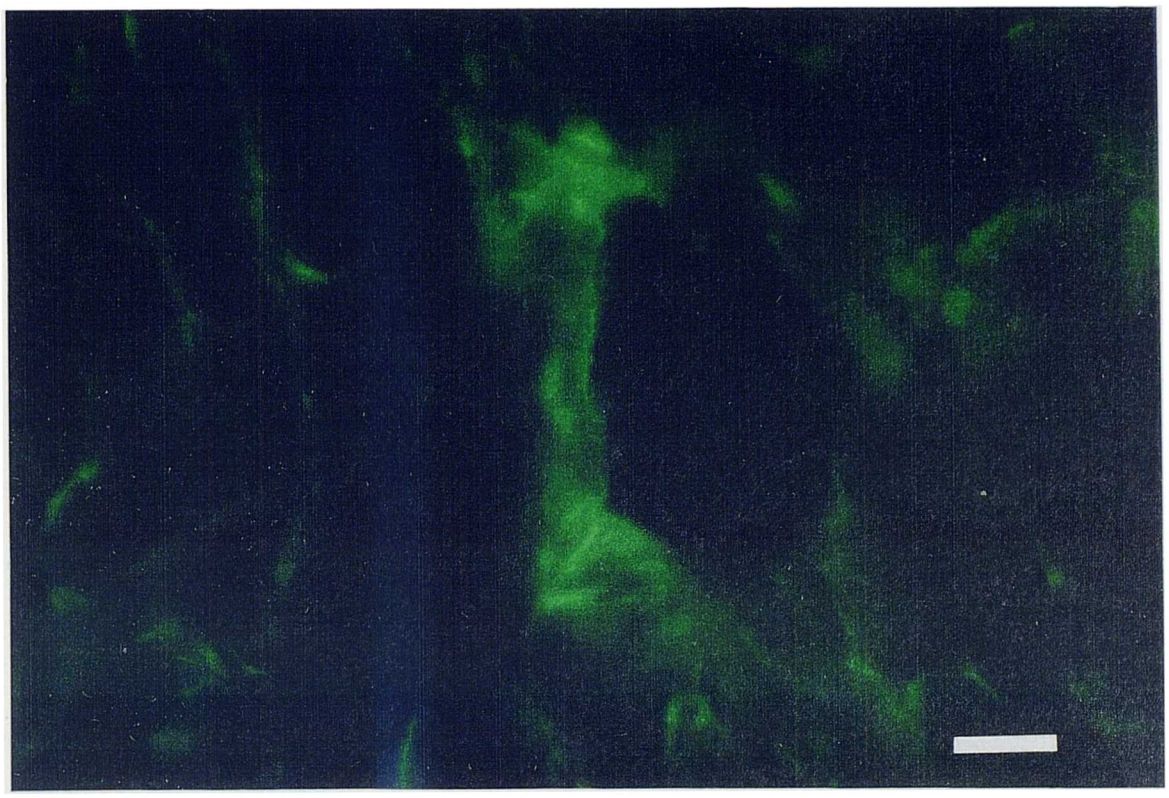


Plate 3.5 Photomicrograph showing intense fibrin immunolocalisation around blood vessels. Bar: 100 μm

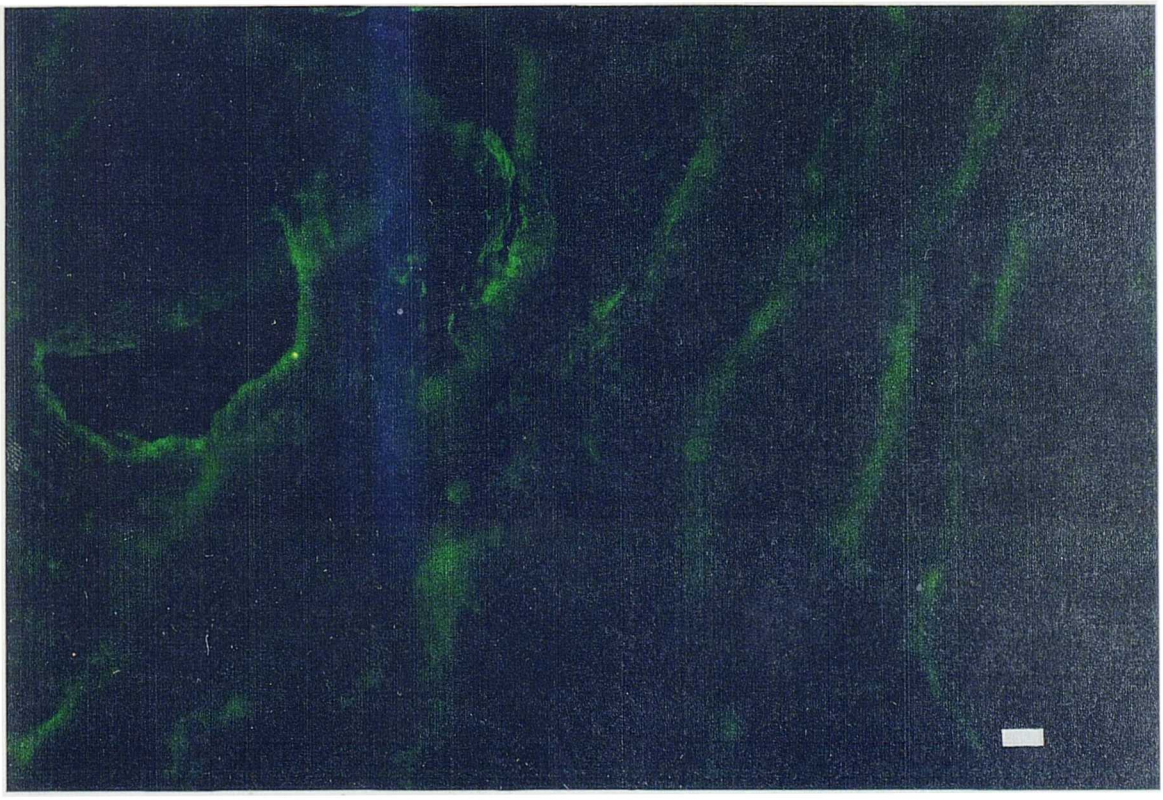


Plate 3.6a Photomicrograph of collagen type I immunolocalisation in the upper dermis. Bar: 100μm

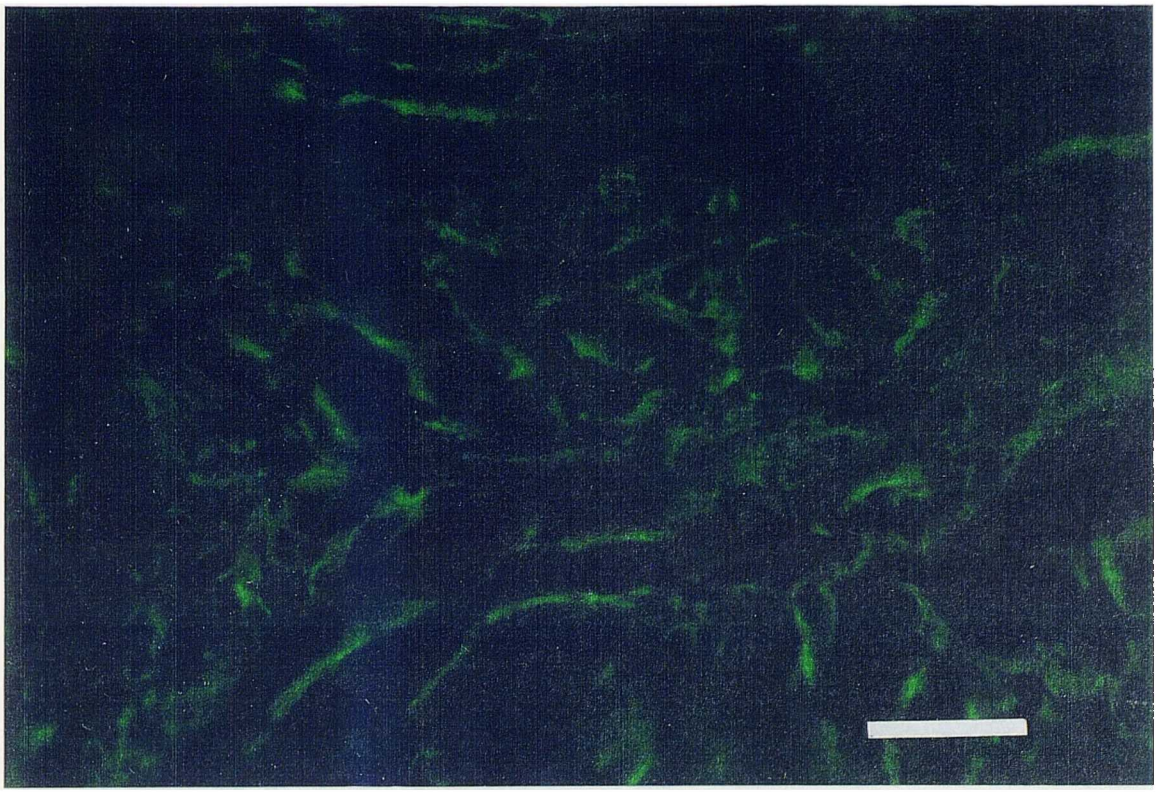


Plate 3.6b Photomicrograph of collagen type I immunolocalisation in the lower dermis. Bar: 100µm

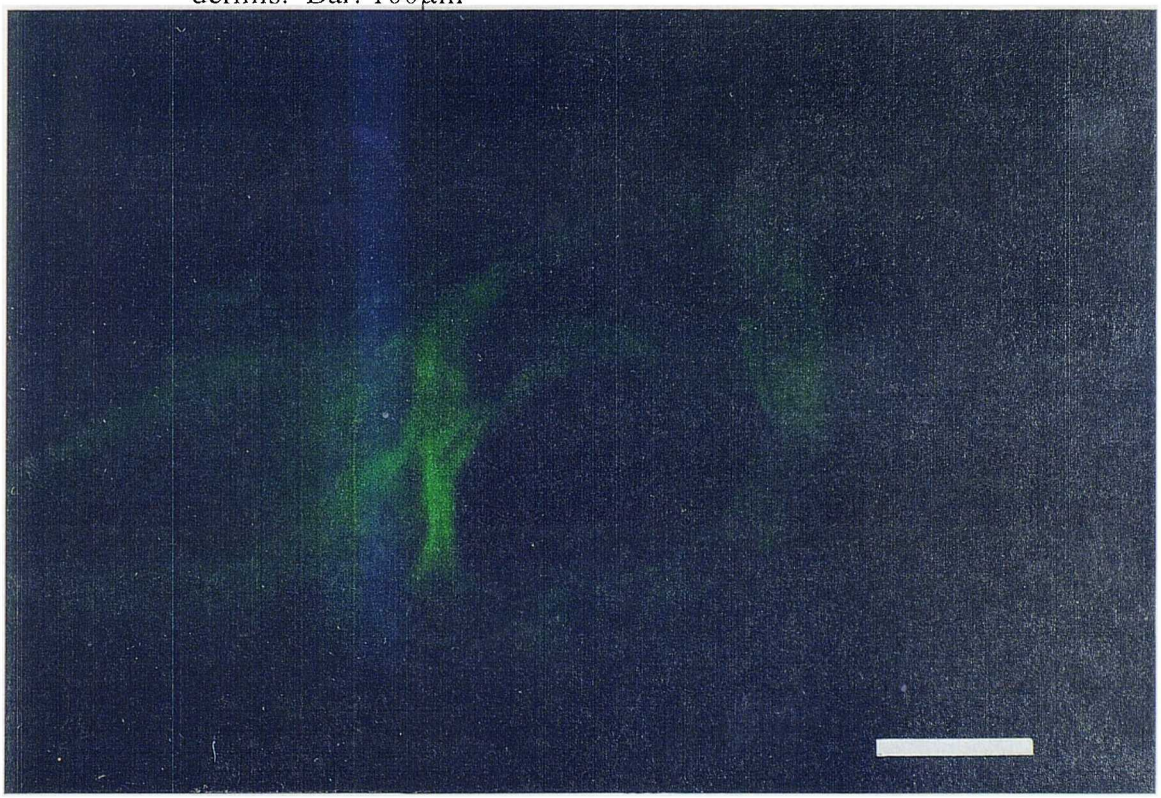


Plate 3.6c Photomicrograph of collagen type I displaying a cuffed appearance around blood vessels. Bar: 100µm

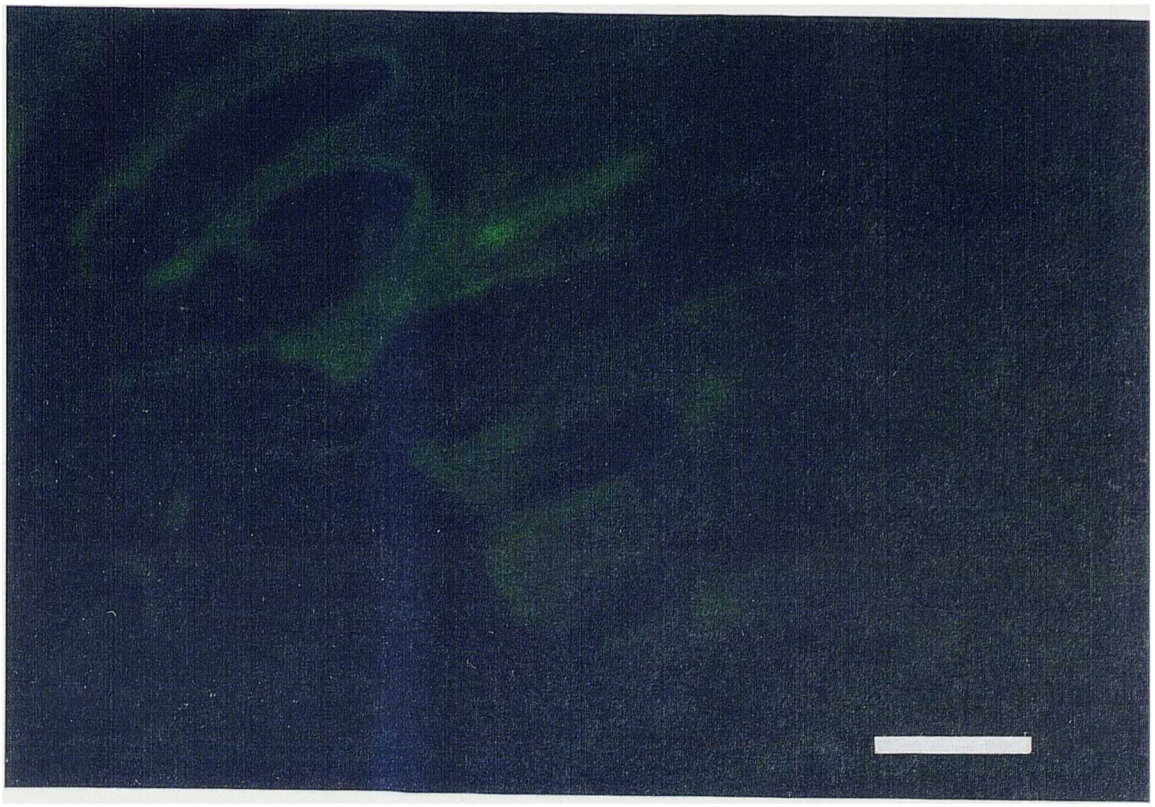


Plate 3.7a Photomicrograph of collagen type IV localised in the epithelial basement membrane. Bar: 100μm

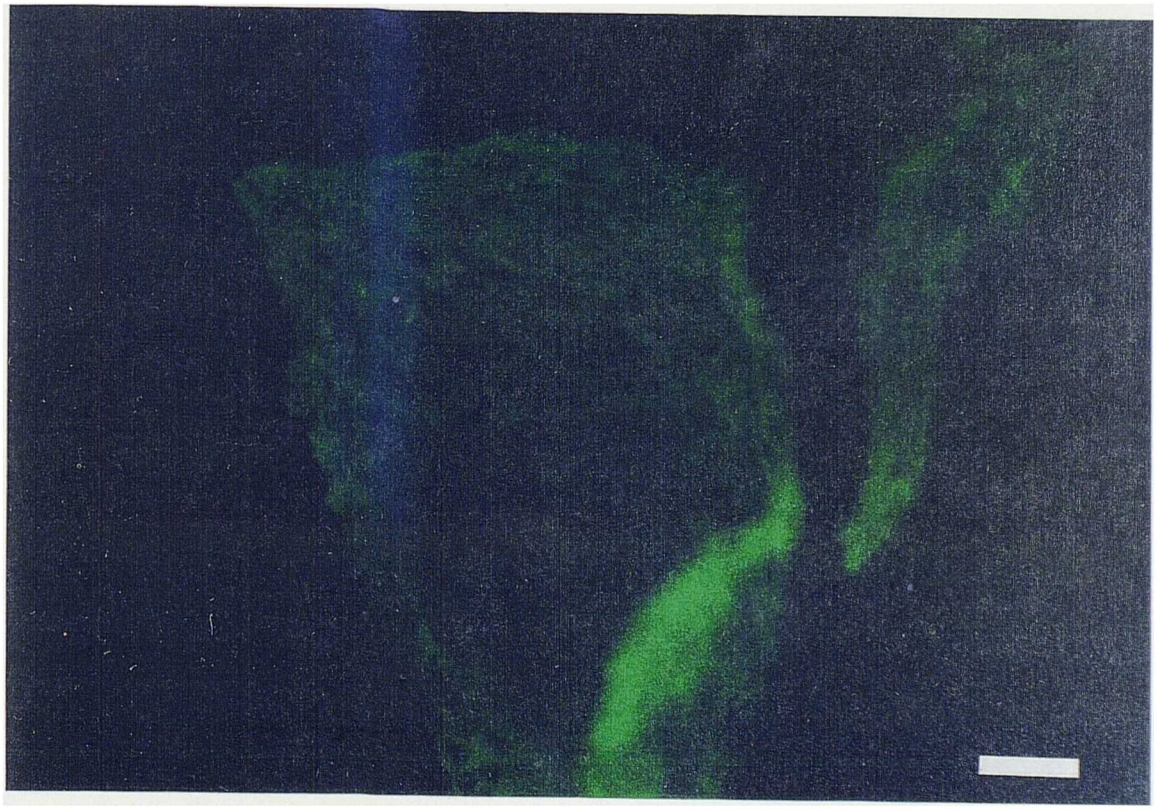


Plate 3.7b Photomicrograph of collagen type IV localised in sweat and sebaceous glands. Bar: 100μm

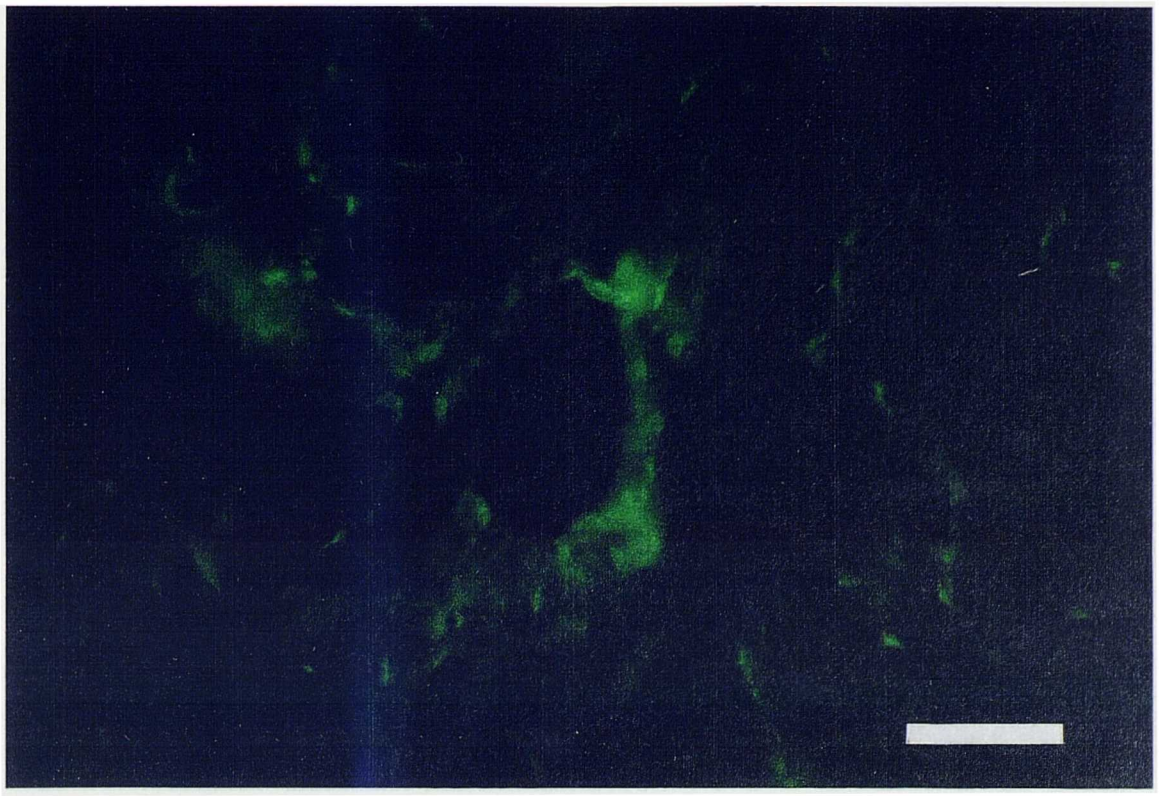


Plate 3.7c Photomicrograph of a blood vessel in the deep dermis that showed intense staining for collagen type IV. Bar: 100μm

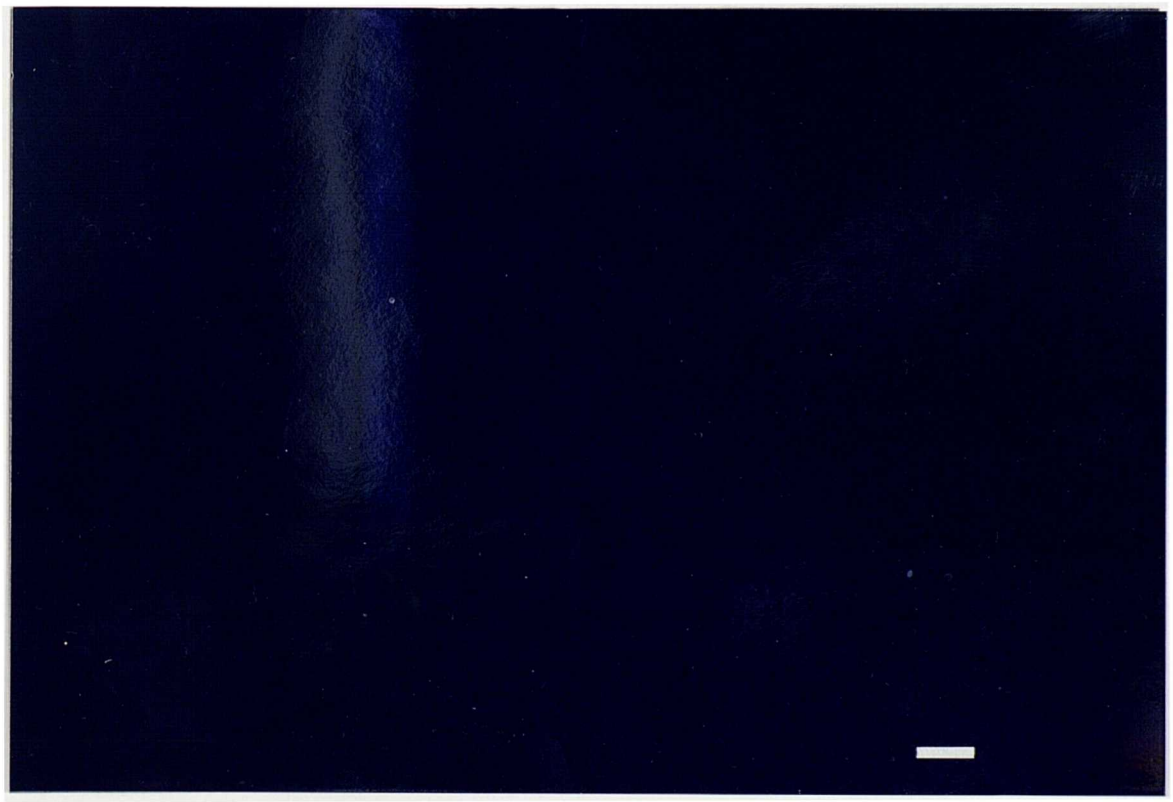


Plate 3.8a Photomicrograph of E-Selectin immunolocalisation in skin sections. Bar: 100μm

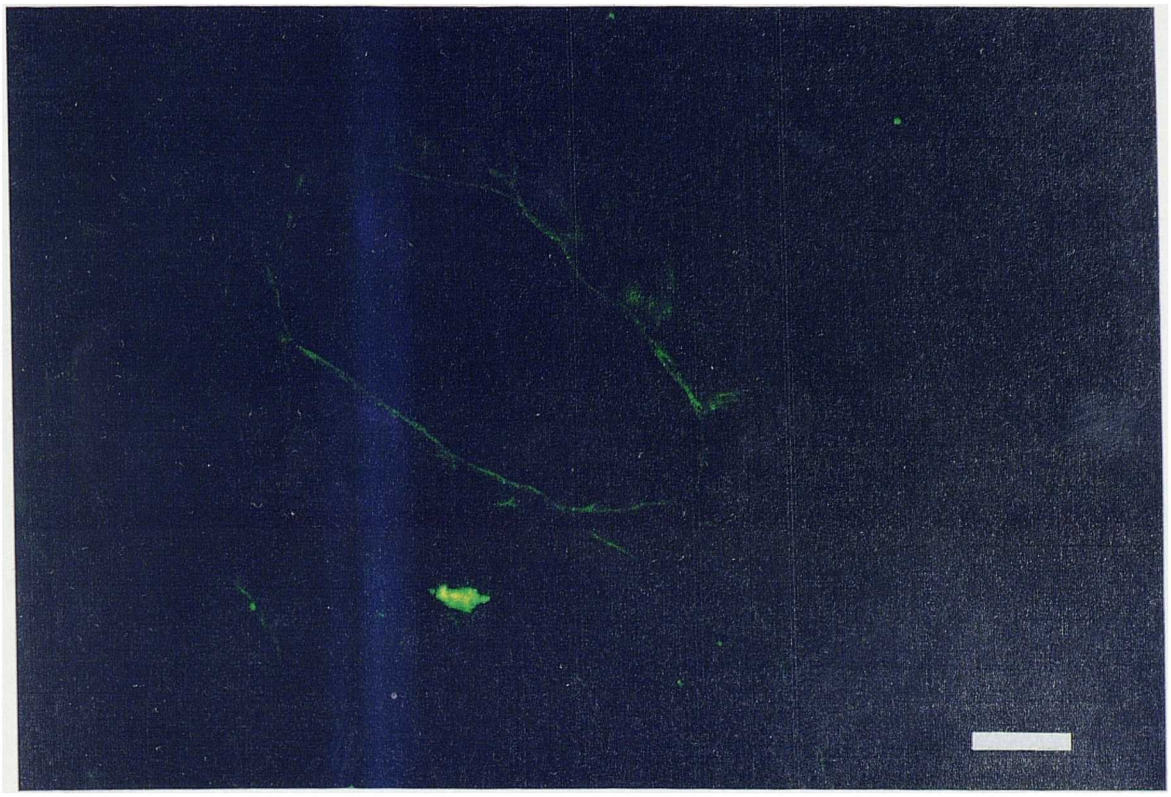


Plate 3.8b Photomicrograph showing E-Selectin immunolocalisation around blood vessels. Bar: 100µm

B) VCAM-1 (Vascular cell adhesion molecule-1)

Staining with this adhesion molecule was consistent and staining was weak throughout the wound area, suggesting that fibroblasts and possibly inflammatory cells contained VCAM-1.

C) ICAM-1 (Intercellular cell adhesion molecule-1)

ICAM-1 was present in the granulation tissue in the wound area. Capillary endothelial and perivascular cells, inflammatory cells and fibroblasts all expressed ICAM-1. Staining was of the same intensity as E-Selectin.

D) PECAM (Platelet endothelial cell adhesion molecule)

PECAM expression was evenly distributed throughout the wound area, particularly in blood vessels. The intensity of the tissue staining was the same as with E-Selectin.

3.3.4 Growth factor distribution during equine wound healing

A consistent pattern of staining with the selected growth factors, was seen in all of the tissue sections. The intensity and distribution of the staining was unchanged wound age.

A) EGF- Epidermal growth factor

Staining with the EGF antibody was seen throughout the epidermis and dermis but was more intense in the wound area (Plate 3.9).

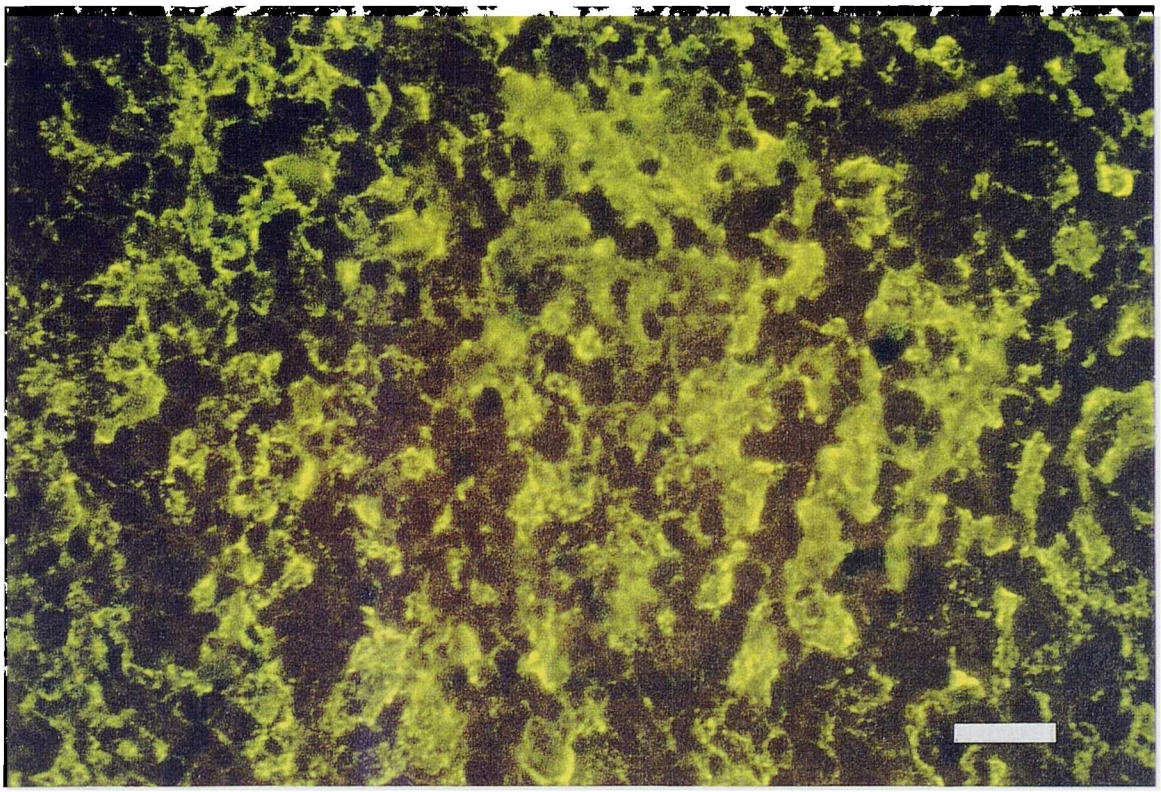


Plate 3.9 Photomicrograph showing EGF immunolocalisation in the dermal tissue. Bar: 100μm

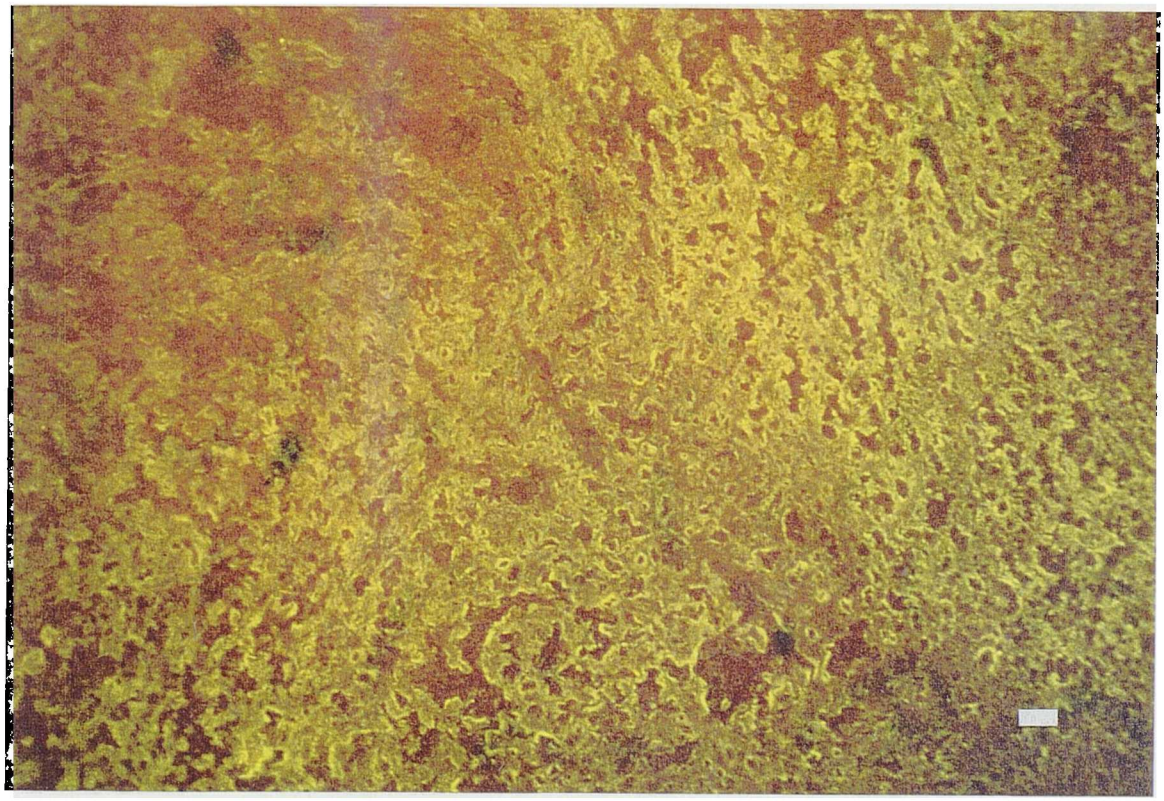


Plate 3.10a Photomicrograph showing FGFa immunolocalisation throughout the wound area. Bar: 100μm

B) FGF (acidic and basic) - Fibroblast growth factor

There was extensive extracellular staining throughout the wound area with FGFa (Plate 3.10a) and FGFb (Plate 3.10b). There was also localised staining of the epidermal layers and basement membrane (not shown). Basic FGF expression was less intense in the wound tissue than the acidic FGF.

C) PDGF - Platelet derived growth factor

PDGF was detected in the epidermis and the basement membrane of the surrounding skin, but the staining was most intense in fibroblasts, inflammatory cells (Plate 3.11) and around blood vessels within the wound area.

D) TGFβ - Transforming growth factor-beta

TGF β 1,2,3 was present in the dermal wound area, epidermis (Plate 3.12a) and the basement membranes. There was also intense staining associated with blood vessels (Plate 3.12b). TGFβ1 showed intense staining at the wound edge (Plate 3.12c). TGFβ2 showed similar staining to TGFβ1 but showed more intense staining around blood capillaries at the wound edge (Plate 3.12d).

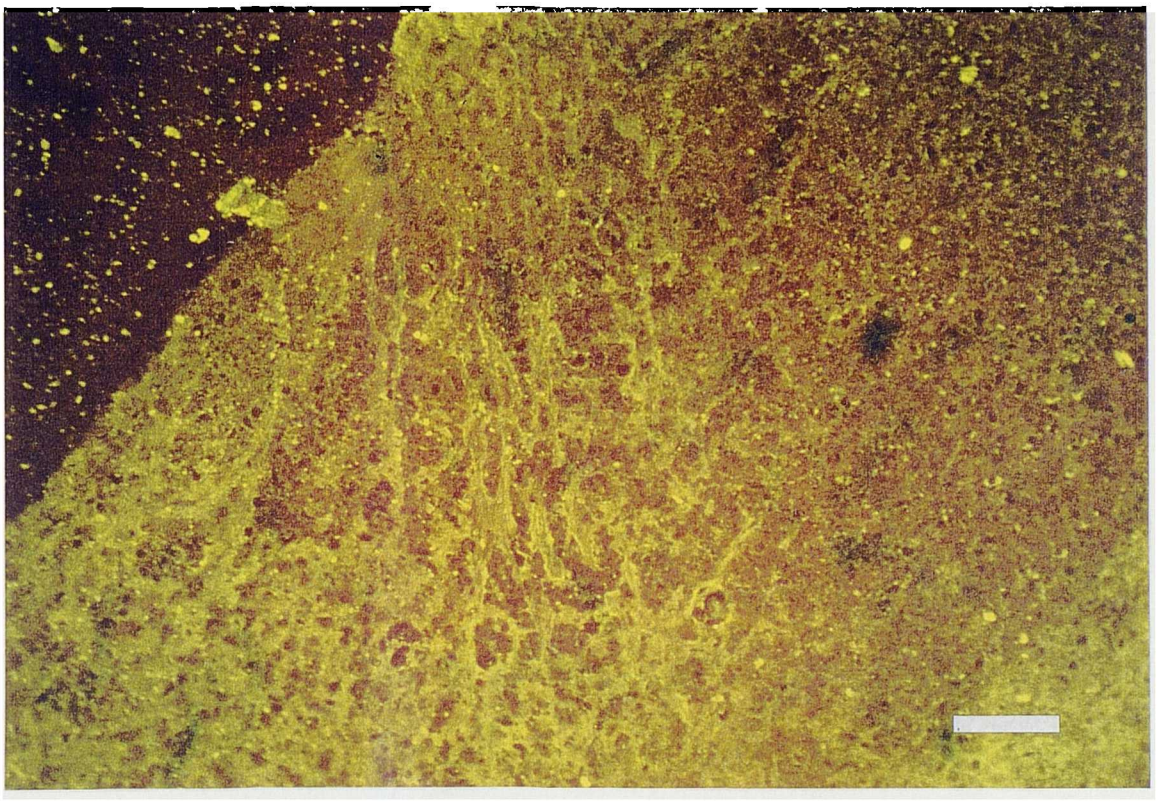


Plate 3.10b Photomicrograph showing FGFb immunolocalisation in the wound area. Bar: 100µm

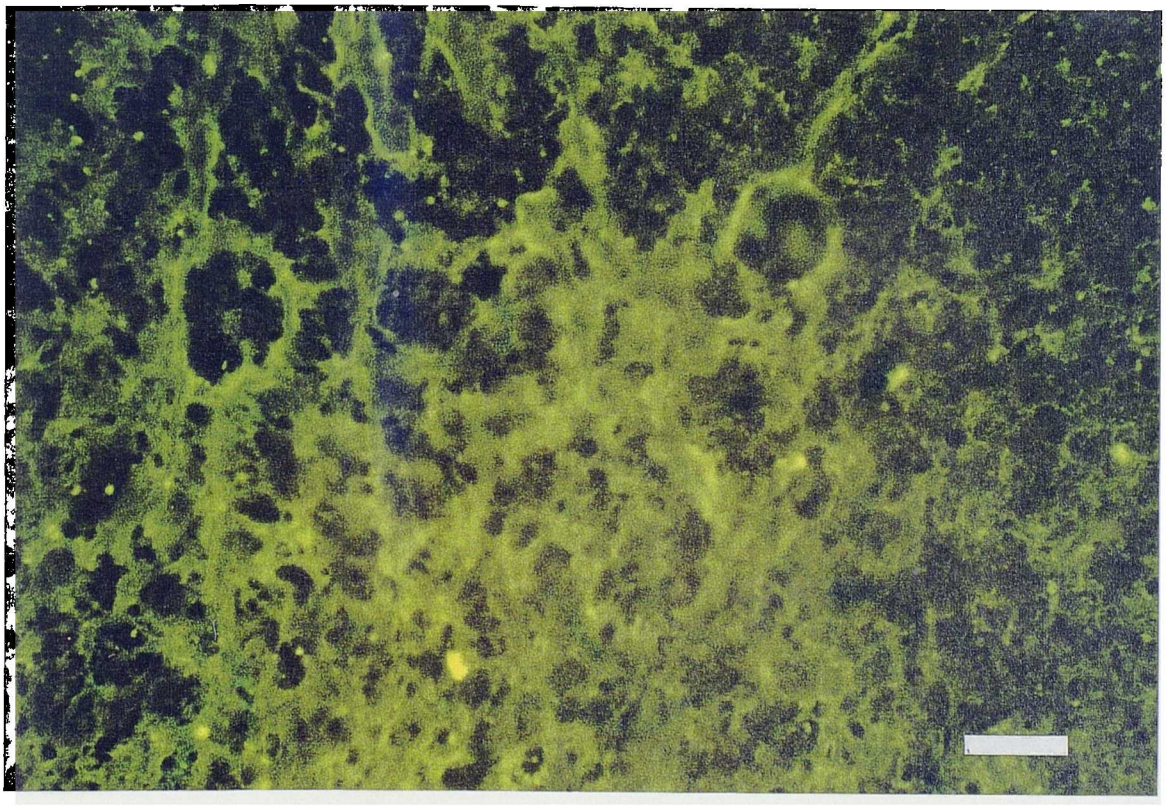


Plate 3.11 Photomicrograph showing PDGF immunolocalisation in the wound area. Bar: 100µm

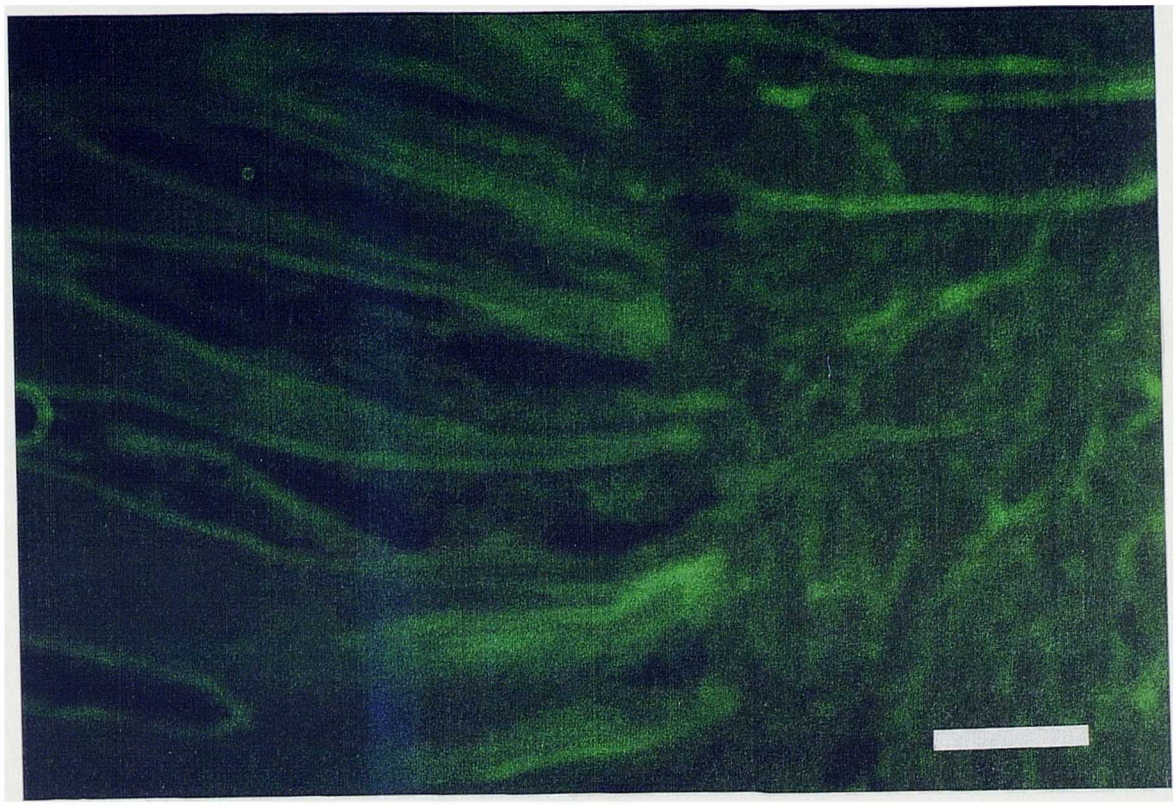


Plate 3.12a Photomicrograph showing the presence of TGFβ in the epidermis and basement membrane. Bar: 100μm

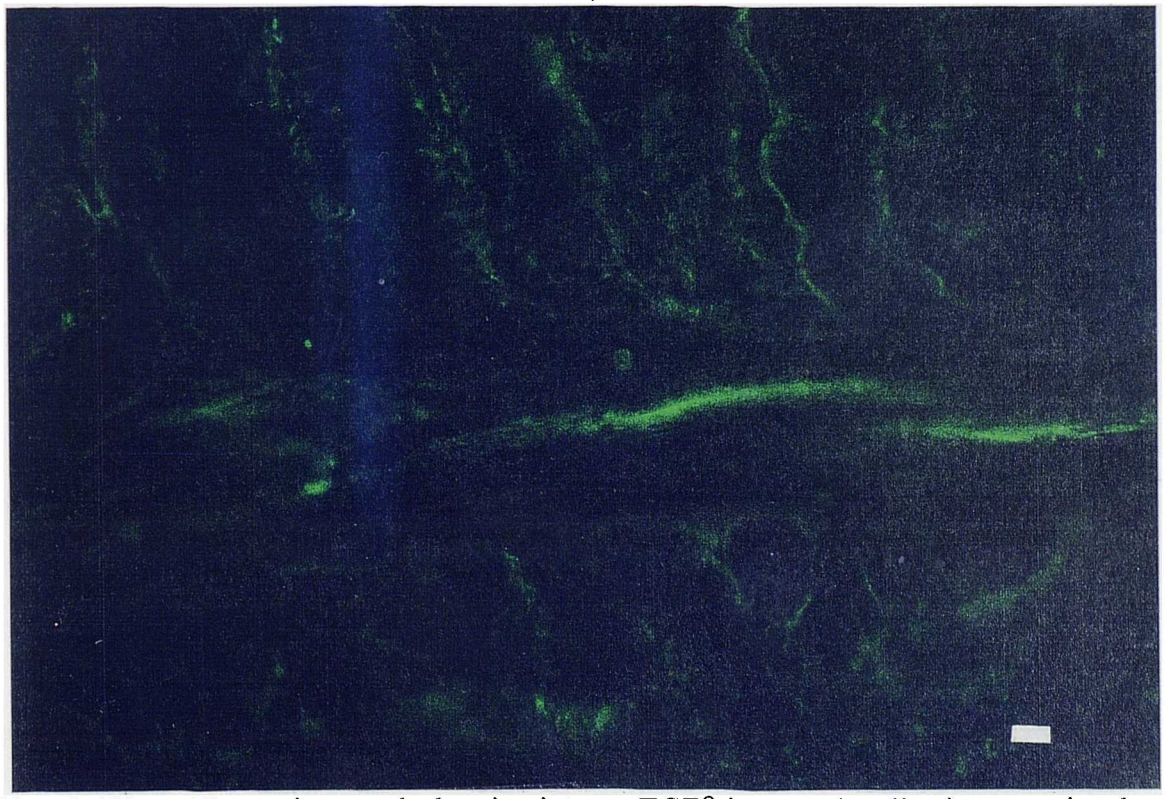


Plate 3.12b Photomicrograph showing intense TGFβ immunolocalisation associated with blood vessels. Bar: 100μm

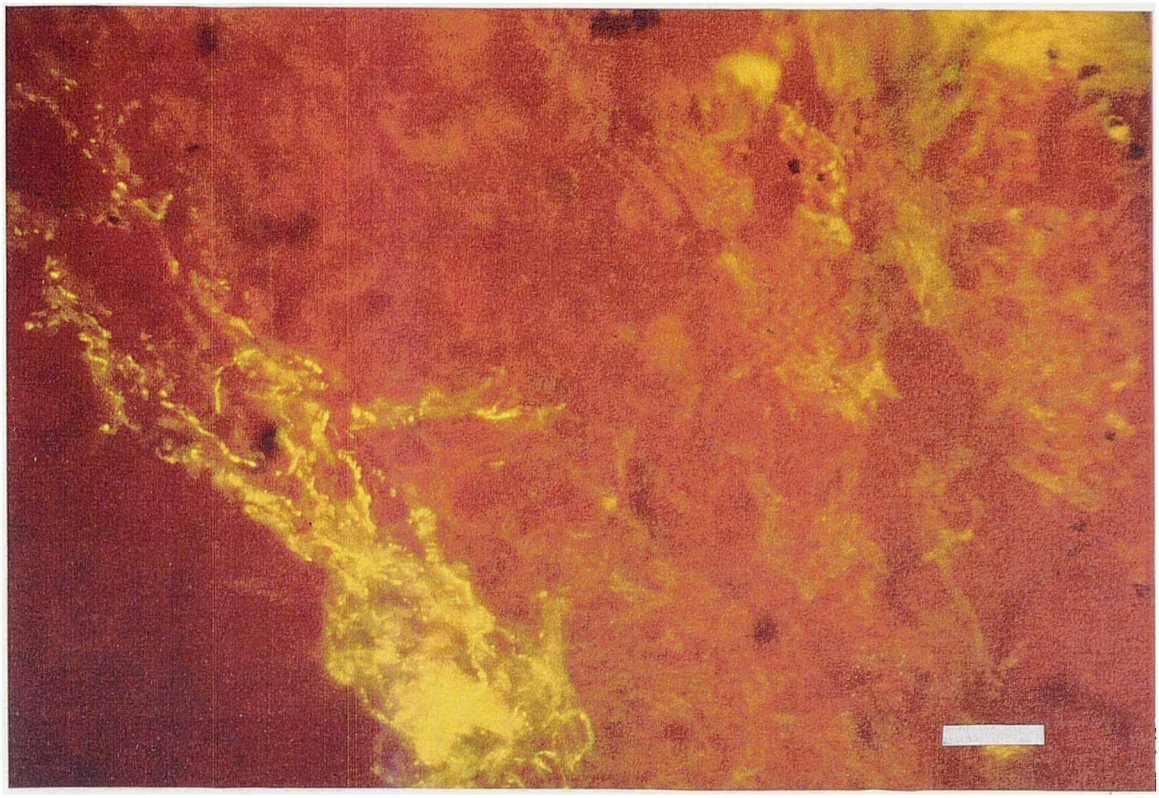


Plate 3.12c Photomicrograph showing intense TGFβ1 staining at the wound edge. Bar: 100μm

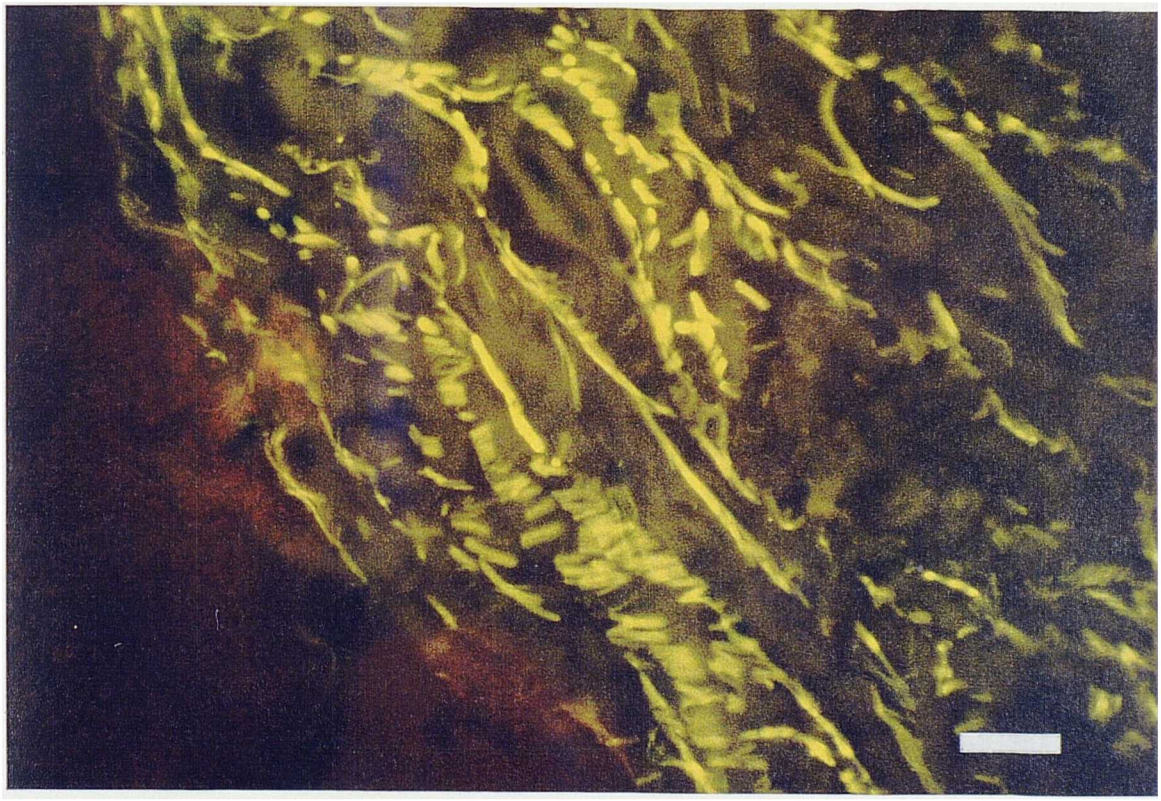


Plate 3.12d Photomicrograph showing intense staining to TGFβ2 around blood capillaries at the wound edge. Bar: 100μm

3.4 Discussion

The mechanisms that control the rate of growth and the adhesion of epithelial cells to granulation tissue are poorly understood. Problems with migration of epithelial cells may be associated with substrate deficiencies or suppressed integrin expression. Evidence from this study suggests that reduced levels of fibronectin within the granulation tissue of the wound may lead to the inhibition of epithelial migration. Fibronectin has many potential roles in wound healing (Clark, 1988). It acts as a substratum for cell migration (Clark *et al.*, 1982a) and a provisional matrix for extracellular matrix assembly (Kurkinsen *et al.,* 1980; Grinnell *et al.*, 1981; McDonald *et al.*, 1982). Lack of fibronectin in the wound area may therefore result in delayed wound closure.

3.4a Pericapillary cuffs

The cuffs surrounding blood vessels were found to be highly organised structures, containing fibrin, laminin, fibronectin, collagen I and IV and endothelial cell adhesion molecules. Fibrin has previously been found surrounding the dermal capillaries of human patients suffering from venous leg ulcers (Wenner *et al.*, 1980) and in skin adjacent to leg ulcers (Leach, 1984).

Claudy *et al*, (1991) using specific antibodies, showed that fibrin cuffs in venous ulcers were mainly composed of undegraded fibrin and not fibrinogen or fibrinogen degradation products. The results from this study and others (Neumann & Van den Broek, 1991; Leu 1991; Highly *et al.*, 1992) have found that these cuffs are not just composed of fibrin. It has been reported that the capillary walls in chronic wounds and venous ulcers were markedly thickened with increased cellularity and extracellular matrix material (Pardes

et al., 1990). The increased cellularity was characteristic of smooth muscle cell, not endothelial cell hyperplasia and the increased extracellular matrix molecules were mainly laminin and type IV collagen, but not usually fibrin or fibronectin. Different tissue preparation methods, staining protocols and antibody specificities may explain the varying results concerning the composition of these pericapillary cuffs.

In this study, the results suggest that the pericapillary cuffs are composed of extracellular matrix molecules. Similar results were found by Herrick *et al.*, (1992) who suggested that the cuffs were composed of extracellular matrix molecules and were not formed by the accumulation of plasma proteins leaked through the vessel walls but were actively assembled by the adjacent connective tissue cells.

3.4b Endothelial cell adhesion molecules

Equine wounds showed elements of inflammation with fibroblast and inflammatory cell infiltration. Also, endothelial cell adhesion molecule expression was evident. As endothelial cell adhesion molecules are expressed at sites of inflammation and injury, it is not surprising that endothelial cell adhesion molecules were present within the granulating wounds. Wilkinson *et al.*, (1993), recently demonstrated that the predominant infiltrating cell types present in lipodermatosclerotic skin of venous disease were T-lyphocytes and macrophages and the expression of E-selectin and VCAM were not elevated, but ICAM-1 expression did increase in more severly diseased skin.

Previous studies have also indicated that E-selectin is persistently present in certain forms of chronic cutaneous inflammation (Groves *et al.*, 1991; Koch *et al.*, 1991). In addition, several groups have suggested the E-selectin may be functioning as a skin specific vascular communicator for memory T cells responsible for organ specific

homing (Picker *et al.*,1991). The inconsistencies in E-selectin expression in acute and chronic inflammation may be due to pulses of endothelial activation throughout the chronically inflamed tissue resulting in the impression of continual expression by individual cells.

VCAM-1 has been previously localised to endothelial cells, resident leucocytes and occasionally a subpopulation of infiltrating leucocytes in inflamed tissue (Griffiths *et al.*, 1991; Norris *et al.*, 1991; Rice *et al.*, 1991;).

In inflamed tissue, many cell types, including endothelium, macrophages, some dermal fibroblasts, (Koch *et al.*, 1991) as well as basal keratinocytes (Griffiths *et al.*, 1991; Norris *et al.*, 1991) express ICAM-1 and PECAM is known to be present around blood vessels in normal and inflamed skin (Albelda *et al.*, 1991). Therefore, the distribution of ICAM-1 and PCAM in normal skin and chronic equine wounds is in agreement with previous reports.

In normal uninflamed skin, endothelial cells express ICAM-1 weakly (Swerlick *et al.*, 1991) and display no immunoreactivity for E-selectin or VCAM-1 (Rice *et al.*, 1991; Griffiths *et al.*, 1991). The observations from this study are in disagreement with these findings. This may be due to differences in staining patterns, which may be the result of differing specificities and affinities of antibodies used and differences in tissue preparation and staining protocols such as cryosectioned or wax embedded material.

3.4c Growth factors

In addition to endothelial cell adhesion molecules, growth factors were also consistently present in the wound area. There are numerous potential sources of growth factors in wounds.

EGF is released by platelets and acts locally during the inflammatory phase in early wound healing. EGF was located consistently in the wound area during this study. Epidermal growth factor promotes healing by stimulating the migration and division of epithclial cells and by increasing the synthesis of proteins, such as fibronectin, which aid in cell attachment and migration. Although, EGF does not induce synthesis of mRNA for extracellular matrix proteins it increases the numbers of fibroblasts in wounds through chemotaxis and mitosis, which results in more total protein production (Bennett & Schultz, 1993).

FGF is often stored bound to heparan sulphate on the cell surface or bound to heparan sulphate proteoglycan such as syndecan in the extracellular matrix and basement membrane (Vlodavsky *et al.*, 1987b). It can be released from these storage sites by heparanase, expressed by activated platelets and neutrophils (Ishai-Michaeli *et al.*, 1990).

The presence of PDGF within the wound corresponds with the release of cytokines from α-granules of platelets during the inflammatory process. Sustained release in the wound may be from macrophages and endothelial cells. Ansel *et al.*, (1993), found no evidence of PDGF polypeptide expression in the epidermis which was consistent with my findings in chronic wounds.

The α-granules of platelets and macrophages are a potent source of TGFβ (Assoian *et al.*, 1987; Wakefield *et al.*, 1987). In addition, TGFβ induces its own expression (Van Obberghen-Schilling *et al.*, 1988) and is a chemoattractant for macrophages resulting in their accumulation in the wound thus perpetuating the raised growth factor levels (Bennett & Schultz, 1993). In the extracellular matrix, TGFβ may be released from several soluble and membrane bound proteins (α-2-macroglobuin,

fibronectin, decorin, type IV collagen and thrombospondin) which are not directly involved in signal transduction (Miyazono *et al.*, 1993). Kane and co-workers (1991), found TGFβ1 expression in the leading edge of migrating epidermis, blood vessels and in cellular infiltrate in the papillary dermis of wounded human skin. TGFβ1 was located at the wound edge in this study, suggesting that its expression was in the cellular infiltrate and blood vessels of the wounded skin. It has more recently been suggested by Levine *et al.*, (1993), that TGFβ 2 and 3 expression was much more intense in the migrating epithelium of wounded tissue than TGFβ1. These findings were also consistent with this study when TGFβ1,2,3 was localised in the epidermis. In humans, TGFβ3 was expressed in the epidermis of intact skin and in that of acute and chronic wounds (Schmid *et al.*, 1993). In the same study, but not detected in intact skin, TGFβ1 mRNA expression was observed in the regenerating epidermis of acute wounds but was not found in chronic wounds. In addition, TGFβ2 mRNA was not detected in the epidermis of any human skin or wound biopsies. Previous localisation of TGFβs in chronic wounds showed TGFβ1 and 2 restricted to the perivascular connective tissue cells in the wound base and edge (Highly *et al.*, 1992).

As in chronic wound healing the release of this growth factor may be due to the inflammatory response. Evidence from my study and that of others suggests that chronic wounds are not deficient in growth factors as suggested by some other investigators (Schultz *et al.*, 1991; Grinnell *et al.*, 1992). It is not known whether the growth factors in chronic wounds are active or whether cells have the ability to respond to them. Immunocytochemistry does not distinguish between intact or degraded growth factors or between active or inactive variants of the same growth factor. Growth factor receptor

levels may be low or of a different receptor isoform type. Low levels of growth factor receptors may occur as a down regulation event in response to high levels of growth factors present or may be degraded by proteases.

Inflammatory cytokines released from activated leucocytes are chemotatic to and increase the proliferation of vascular smooth muscle cells and dermal fibroblasts. Therefore, fibroblasts may migrate into blood vessels, where cytokines stimulate the localised synthesis and inhibit the degradation of extracellular matrix molecules such as laminin, fibronectin, collagen I and III. Fibroblasts, smooth muscle cells and endothelial cells may all actively deposit matrix molecules around capillaries in a complex cuffed pattern. Fibrin which initially leaks from the permeable blood vessels may provide a framework for extracellular matrix synthesis, to trap active cytokines and is known to be a chemoattractant for fibroblasts. The presence of cuffs may be to protect delicate capillaries and limit the leakage of large molecules from these blood vessels.

3.4d Equine wounds

Once chronic equine wounds are formed they may persist in the absence of therapeutic measures for years. The reasons why this delayed healing occurs at one common anatomical site (lower limb) may relate to the sufficiency and extent of local vascular networks, thinness of skin causing tissue tension and the presence or absence of deeper venous return pathways. It is not known if venous insufficiency in the horse exists as the micro circulation of the horse has not yet been documented.

The results from this study suggest that chronic equine wounds are composed of an unhealthy granulation tissue which may be lacking in essential extracellular matrix molecules such as collagen and other proteins, needed for epithelisation and wound

healing. Work in the next chapter describes the biochemical analysis of the composition of normal skin, granulation and sarcoid tissue to establish the distribution of these important molecules.

CHAPTER FOUR

The examination of extracellular matrix molecules in sarcoid, granulation tissue, normal skin biopsies by comparative biochemistry

4.1 INTRODUCTION

There have been no immunochemical or biochemical studies investigating the distribution of extracellular matrix molecules in equine wounds, although there have been many such studies examining extracellular matrix distribution in acute wounds in man and other animals. These immunochemical studies form an important initial analysis of extracellular matrix distribution. Biochemical analysis gives a more quantitative interpretation of protein presence or absence in wound tissue.

Many biochemical studies have been performed by implanting cellulose sponges or teflon tubes (ePTFE) within the skin of animal models as well as human volunteers and measuring the degree of inflammation, fibroplasia and collagen deposition after removing the implant (Goodson & Hunt, 1984; Jensen *et al.,* 1986; Goodson, 1987; Haydock & Hill, 1987). In these wounds, granulation tissue matrix molecules were deposited in an ordered sequence of fibronectin, type III collagen and type I collagen (Gay *et al.,* 1978; Kurkinsen *et al.*, 1980). The relevance of these studies to chronic wounds is uncertain because the normal repair process is arrested either initially or at some stage during its development.

Steroid-treated, diabetically-induced and surgically manipulated animal wound models have been designed to mimic chronic wound healing (Burnand *et al.*, 1982; Pierce *et al.*, 1989a; Knighton *et al.*, 1990; Bernstein *et al.*, 1991; Mustoe *et al.*, 1991; Pierce *et al.*, 1992). These models have restricted application since they do not reproduce any underlying problems with blood supply, therefore any investigation of chronic wound healing has to be restricted to the individual patient. This in turn, restricts what can be done experimentally in terms of monitoring wound healing processes such as extracellular matrix synthesis in the equine species.

Biopsies obtained from granulating wounds, sarcoids and normal healthy skin (at post mortem) represent an important opportunity to study quantitatively the complex distribution of extracellular matrix molecules found in chronic equine wounds. Therefore, using both established and novel biochemical techniques, the protein, collagen and TGFβ composition of sarcoids, granulating wounds, normal healthy equine skin, was investigated and compared to that found by immunocytochemistry.

4.2 METHODS

4.2.1 Source of biopsy material

A second set of normal skin biopsies was obtained from horses as described in Table 2.1, snap frozen in liquid nitrogen and stored at -80°C until needed.

4.2.2 Dry weight

Each piece of frozen tissue was lyophilised and stored at -70° C until needed (Chapter 2, section 2.2.2).

4.2.3 Protein extraction

Tissue samples were homogenised as previously described in Chapter 2, section 2.2.3.

4.2.4 Protein content of biopsies

The protein content was determined according to the method of Lowry et al, (1951), using folin phenol reagent (BDH), see Chapter 2, section 2.3.4. The total protein of the biopsies was expressed as μg/g dry weight.

4.2.5 Electrophoresis analysis of biopsy proteins

The biopsy samples were analysed by sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE), (Laemmli, 1970). The method is described in Chapter 2, section 2.2.5.

4.2.6 Collagen synthesis

The cold hydroxyproline method of Stegman and Stadler (1967) was used to analyse the collagen content of the lyophilised tissue samples (Chapter2, section 2.2.6). Collagen content of the tissue samples was calculated by assuming that it contains 13.6% hydroxyproline (Shah et al., 1994) and expressed as μg/g of the total biopsy dry weight.

4.2.7 PICP procollagen determination

The PICP assay was performed on the tissue homogenates as described in Chapter 2, section 2.2.7.

4.2.8 TGFβ analysis

The quantities of TGFβ in the tissue homogenate was measured (Chapter 2, section 2.2.8)

4.3 RESULTS

4.3.1 Protein content of tissue samples

The dry weight, water content and total protein of the normal, granulation and sarcoid tissue was analysed (Table 4.1). The granulation tissue biopsies showed a higher protein content than the sarcoid and normal tissue biopsies (μg protein), however this increase was not significant. The sarcoid biopsies showed a lower protein content than the normal tissue. When the results were expressed as (μg protein/g dry weight) the granulation tissue biopsies still showed a higher protein content. However, sarcoid

tissue biopsies had a slightly higher protein content than the normal skin but a lower protein content than the granulation tissue. These differences from the normal skin were not significant. This was due to the fact that the mean biopsy weights of the sarcoid tissue was lower than the normal and granulation tissue samples.

4.3.2 Electrophoretic analysis of biopsy proteins

The proteins present in the biopsies were solubilised in 2M NaCl, 50mM phosphate buffer, precipitated with TCA and separated by SDS-PAGE (Plate 4.1). Samples from normal and granulation tissue showed a similar protein profile when subjected to electrophoresis. The sarcoid tissue however, revealed far fewer protein bands than the granulation and normal tissue (Plate 4.1). All of the samples (normal, granulation and sarcoid) showed polypeptide bands of 70kd (highest intensity in the granulation tissue and lowest in the sarcoid), 59kd (lowest intensity in the sarcoid) and 26kd (very low in sarcoid). The granulation tissue revealed a variety of protein bands ranging from 200-26kd. The normal tissue separated into similar polypeptide bands but these bands were less intense. The polypeptide bands separated from the sarcoid tissue however, were undistinguishable. The staining revealed similar bands to the normal and granulation tissue but they were very indistinct suggesting that there was much less protein present.

Legend: SF - Sarcoid derived tissue; GT - granulation tissue; NF - tissue derived from normal skin; MW - Molecular weight markers

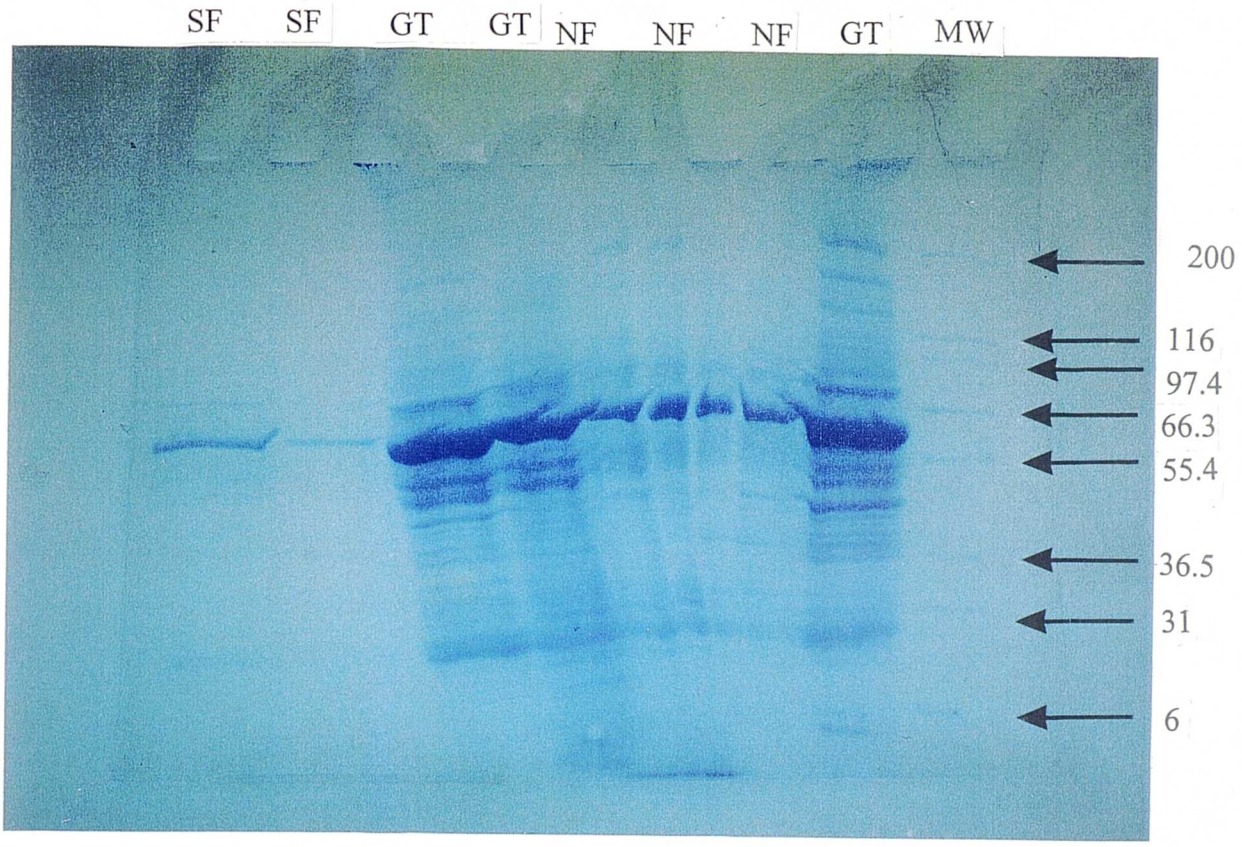


Plate 4.1 Proteins present in biopsy samples from normal skin, granulation and sarcoid tissue

Table 4.1 Total dry weight, water and protein content of sarcoid, granulation tissue and normal skin biopsies.

Tissue type	Dry Weight (g)	Water Content (g)	Total Protein (μg)	Protein (μg/g dry weight)
normal tissue (n=18)	0.051±0.004	0.125±0.012	389.7±94.4	7641
granulation tissue (n=15)	0.045± 0.008	0.173±0.036	434.1±69.9	9646
sarcoid tissue (n=12)	0.039±0.008	0.121±0.025	354.0±69.8	9077

To calculate the protein content skin biopsies were solubilised in 50mM phosphate buffer, 2M NaCl and analysed for protein according to Lowry *et al.*, (1951).

4.3.3 Collagen analysis of skin samples

The hydroxyproline and calculated collagen content of the normal, granulation and sarcoid skin biopsies is shown in Table 4.2. The granulation tissue contained significantly more ($p<0.0001$) hydroxyproline (μg/g dry weight) than the normal and the sarcoid tissue ($p<0.0001$). The level of hydroxyproline (μg/g dry weight) in the sarcoid skin was much lower than the normal ($p<0.0001$) and granulation skin biopsies ($p<0.0001$).

The collagen content (μg/g dry weight), in the normal skin was slightly higher than in the granulation tissue skin biopsies ($p<0.0001$). In the sarcoid skin the collagen content (μg/g dry weight) was much lower than the granulation tissue and the normal skin ($p<0.0001$).

Table 4.2 Hydroxyproline and percentage collagen content of normal, granulation and sarcoid skin biopsies.

Tissue type	Dry weight (g)	Hydroxyproline (μg)	Hydroxyproline (μg/g dry weight)	Collagen (μg/g dry weight)
Normal tissue (n=18)	0.051±0.004	15.2±2.4	297.8	40.6
Granulation tissue (n=15)	0.045±0.008	14.6±2.2	323.6	44.0
Sarcoid tissue (n=12)	0.039±0.008	5.8±0.9	147.7	20.0

Collagen content of the biopsies was calculated by assuming that it contains 13.6% hydroxyproline.

4.3.4 PICP procollagen determination of skin samples

The levels of PICP type I procollagen were measured in normal, granulation and sarcoid biopsies (Figure 4.1). The levels of PICP in the granulation tissue biopsies was significantly higher ($p<0.02$) than in normal skin. There was also a difference between the granulation tissue and sarcoid biopsies in the levels of PICP. Although the levels appeared higher in the granulation tissue than the sarcoid biopsies, this difference was not statistically significant. The normal skin biopsies had however, significantly lower PICP concentrations ($p<0.05$) than the sarcoid skin biopsies.

4.3.5 TGFβ analysis on skin biopsies

The assay was not sensitive enough to measure the TGFβ concentration in normal skin biopsies. Therefore, the levels must be very low in normal healthy skin. The TGFβ concentrations in the granulation tissue biopsies was significantly higher ($p<0.0001$) than in the sarcoid and normal skin biopsies (Figure 4.2).

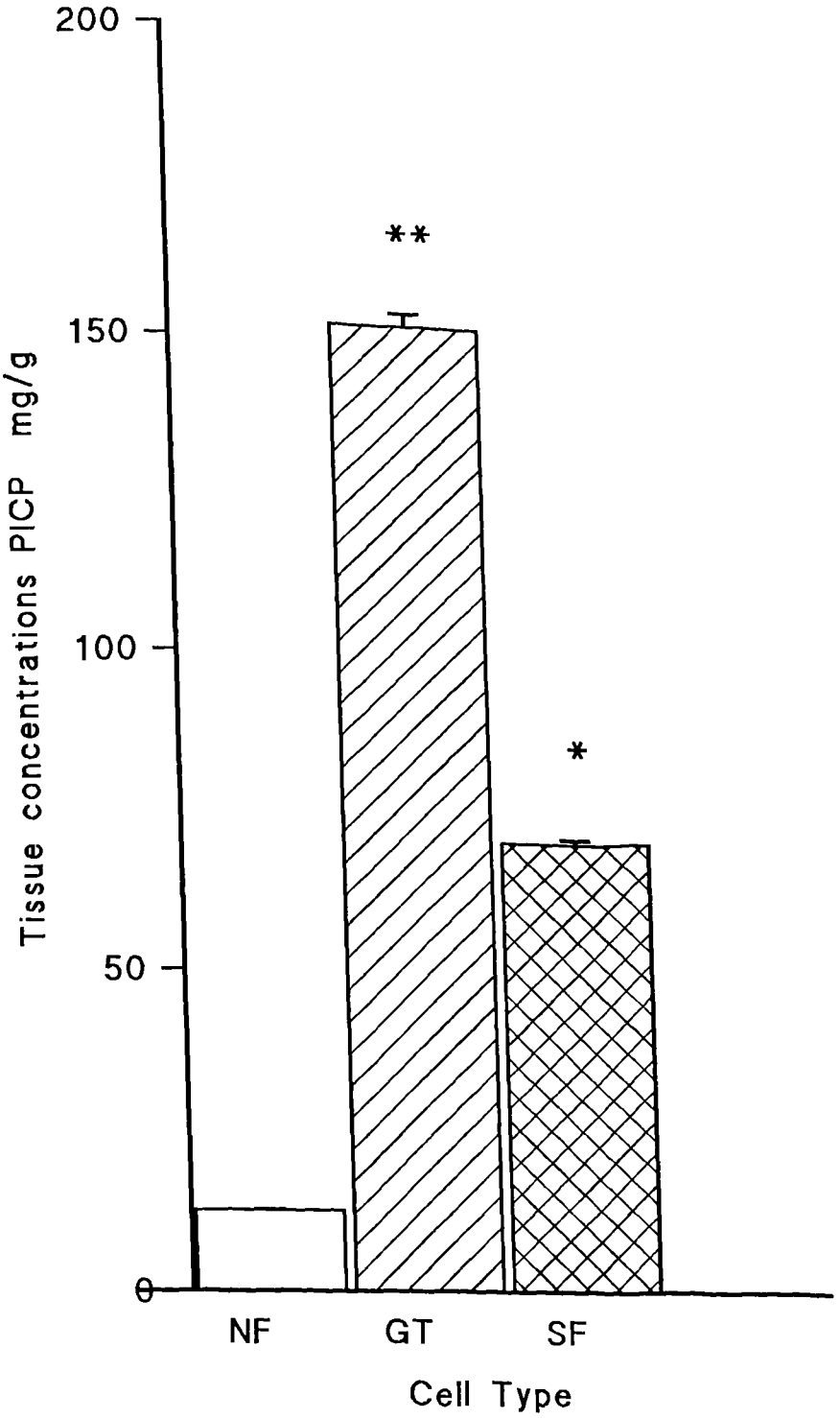


Figure 4.1 Concentration of the carboxyterminal propeptide of type I collagen (PICP) in tissue homogenates of normal (n=6), granulation (n=10) and sarcoid (n=6) skin. Values significantly different to normal skin and granulation tissue ** $P<0.02$; values significantly different to normal skin and sarcoid tissue * $P<0.05$.

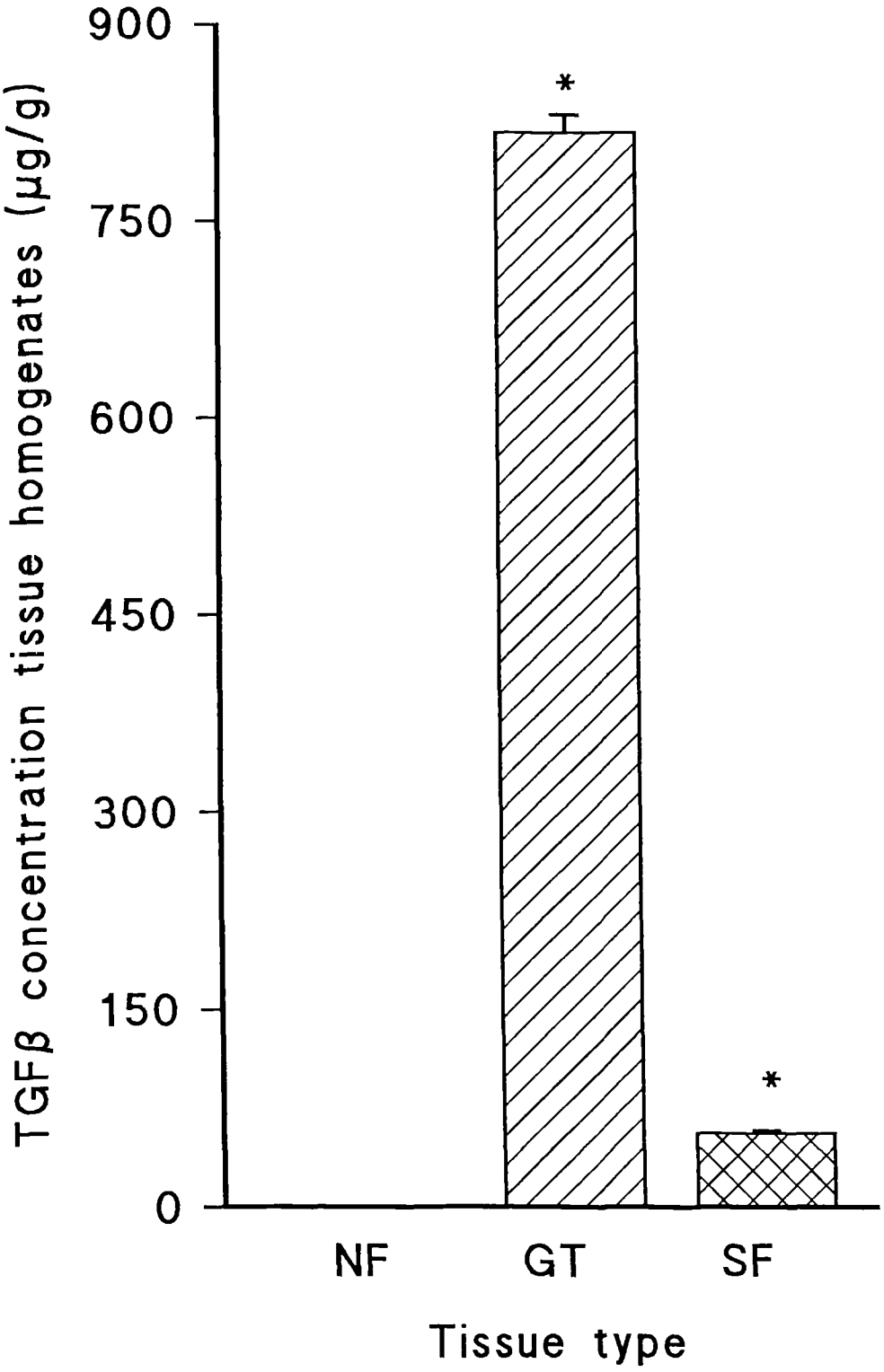


Figure 4.2 TGFβ concenterations were measured in normal skin (n=6), granulation tissue (n=6) and sarcoid tissue (n=6). Values significantly different to normal and sarcoid skin * $p<0.0001$; values significantly different to normal skin and granulation tissue * $p<0.0001$.

4.4 Discussion

The identification and quantification of proteins and extracellular components of the tissues under investigation may give some indication as to the processes occurring during wound healing *in vivo*.

The extracellular matrix composition of granulation tissue from acute and chronic wounds was found to be different to that of normal dermis (Bailey *et al.*, 1975, Clark, 1990). Keloids have raised levels of extracellular matrix molecules, compared to normal granulation tissue (Fleischmajer *et al.*, 1980; Rockwell *et al.*, 1989). Initially, during normal wound healing proteases are released in association with the clotting cascade. Also additional matrix degrading metalloproteinases are secreted by neutrophils and macrophages (Hibbs *et al.*, 1985; Wahl & Wahl, 1985) to remove denatured connective tissue proteins and allow remodelling of the wound by dermal and epithelial cells. These proteases accumulate in wound fluid, collagenase and elastase have been detected in blister fluids from bullous skin diseases (Oikarinen *et al.*, 1983).

Under SDS PAGE gel electrophoresis, the sarcoid tissue displayed less intense protein bands when compared to normal and granulation tissue. This suggests differences in the sarcoid extracellular matrix components. The level of protein was lower in the sarcoid tissue when compared to normal and granulation tissue but, this difference was not statistically significant. The hydroxyproline and collagen levels in the sarcoid tissue were significantly lower than the normal and granulation tissue which possibly explains why the higher molecular weight bands were either missing or very faint on the SDS page gels.

The low levels of collagen found in sarcoid skin compared to normal and

granulation tissue may be related to a decreased number of viable matrix producing fibroblasts within the tissue. This may be due to a) reduced proliferation and migration of viable fibroblasts, b) a decreased production or secretion of matrix molecules by fibroblasts already present within the tissue and c) an increased degradation of the matrix by existing proteolytic enzymes present in the tissue. The matrix constituents of a wound depends on the cells, enzymes and the growth factors present (Ignotz & Massague, 1986; Roberts *et al.*, 1986; Pierce *et al.*, 1989). Environmental influences, such as infection may lead to problems in both cellular and metabolic processes during granulation tissue formation in the chronic wound. Therefore factors directly affecting the normal growth factor and receptor distribution in chronic wounds, may indirectly affect granulation tissue formation and wound closure.

Chronic equine wounds are susceptible to infection and consequently inflammatory cell and bacterial protease production. Wysocki *et al.*, (1991) found that regulation of the protease cascade varied from the normal and metalloproteinases were over expressed in chronic wound fluid from leg ulcers. They found 94kD and 168kD gelatinase released from peripheral blood cells during blood clotting, a 110kd gelatinase released from tissue cells and a 72 kd serum gelatinase in chronic wound fluid. The chronic wound metalloproteinase concentration was 5-19 fold higher than in acute wound fluid (Wysocki *et al.*, 1993). Schultz *et al.*, (1993) also detected an increase in the level of proteolytic activity in chronic wound fluid compared to acute wound fluid. Over production of these metalloproteinases may lead to excessive degradation of pre-existing matrix, newly forming matrix tissue and cytokines and their receptors (Grinnell *et al.*, 1992). A combination of cell blotting, immunoblotting and cell adhesion assays have

been used to analyse fibronectin and vitronectin in wound fluid from acute and chronic wounds (Grinnell *et al.*, 1992). Acute wound fluid contained intact fibronectin and vitronectin as major cell adhesion proteins. Whereas, chronic wound fluid from venous leg ulcers showed a mixture of complete and partial degradation of vitronectin and fibronectin. Fibronectin was degraded into small polypeptide fragments. The fibronectin fragments in the wound bed could promote high levels of proteases by stimulating neutrophil degranulation (Wachtfogel *et al.*, 1988) and fibroblast secretion of metalloproteinases (Werb *et al.*, 1989).

These findings may explain the low levels of proteins observed by SDS-PAGE in the sarcoid tissue. It may be that the proteins (including collagen) in the sarcoid tissue are being degraded by the over expression of proteases before being processed. The chronic wound environment may impair the ability of cells, especially fibroblasts, to synthesise matrix molecules such as fibronectin and collagen. High levels of fibrin deposition have been associated with extracellular matrix accumulation *in vivo* (Dvorak, 1986), however recent investigations by Falanga and Pardes (unpublished, Falanga, 1993) have shown that fibrin and fibrinopeptide B which are found in venous leg ulcers have a direct down regulatory effect on procollagen type I synthesis by dermal fibroblasts in culture. Also, fibroblasts on fibrin gels were found to synthesise less collagen. Therefore, fibrin degradation products, in large quantities may be beneficial in stimulating wound fibroblast migration (Senior *et al.*, 1986) but may be detrimental to extracellular matrix synthesis. Extracellular matrix molecules and the growth factors responsible for stimulating their synthesis may be continually degraded (Raghow *et al.*, 1987; Ignotz *et al.*, 1987).

Increased degradation or impaired synthesis of collagen and adhesive proteins such as fibronectin and fibrinogen, would prevent the formation of healthy tissue necessary for keratinocyte migration and wound closure. If however, collagen and adhesive proteins were not degraded sufficiently, this could lead to the hyper-granulating wounds seen in the equine species. Rasmussen *et al.*, (1992) found that the propeptides of collagen in wound fluid from venous ulcers treated with growth hormone increased significantly during healing. It suggested that both collagen I and III production increased as the wounds healed.

Future work may include investigating the distribution of proteases in granulation and sarcoid tissue in comparison with normal skin. Culturing the cells obtained from granulation, sarcoid and normal skin allows the study of growth and morphology of these cells and also their response to the addition of exogenous growth factors. Due to the difficulty in understanding the process of wound healing in equine skin *in vivo*, it was decided to study the cell culture of normal, granulation tissue and sarcoid skin.

CHAPTER FIVE

Equine dermal and epithelial cell culture

5.1 INTRODUCTION

In the last chapter *in vivo* equine wound healing was discussed. As expermentation on equine wounds is unethical due to the fact that our main interest is the welfare of horses, it was considered that dermal and epithelial cell culture would provide an adequate model for equine wound healing investigations.

In normal wounds the inflammatory response that has been provoked by tissue injury, is rapidly followed by a fibroblastic phase as a significant number of fibroblasts appear at the avascular space (Ross and Benditt, 1961). There is evidence that the wound event selects various fibroblast subtypes causing expression of certain phenotypes from an otherwise heterogenous dermal population (Regan *et al.*, 1991). Although the horse with a hyper-granulating wound provides us with a satisfactory naturally occcuring acute wound model, it is an unacceptable model for experimental monitoring of wound healing. Therefore such investigations were undertaken using tissue derived from them during the course of surgery or post-mortem. Cell culture offers an opportunity to study the morphology and proliferation rates of cells produced in isolation. Furthermore, tissue from different areas of the body along with exuberant granulation tissue and the different types of sarcoids can be cultured and may offer an insight into how regional epithelial differentiation occurs.

The biology of normal dermal fibroblasts, granulation tissue and sarcoid -derived fibroblasts and epithelial cells has been studied. The cells derived from six different sites of the normal horse and three different types of sarcoid have been compared. This chapter deals with the establishment of culture conditions for dermal fibroblasts and epithelial cells and the normal response of these cells to the addition of serum. Emphasis is placed on their morphology and their ability to proliferate in cell culture conditions.

5.2 METHODS

5.2.1 Source, explantation and culture of equine dermal fibroblasts

Dermal Tissue was obtained post mortem from horses and a pony which had been killed for other reasons (Chapter 2, section 2.3.1).

5.2.2 Source, explantation and culture of equine epithelial cells

Equine skin was obtained post mortem, explanted and cultured (Chapter 2, section 2.3.2)

5.2.3 Anti-cytokeratin staining

The epithelial cells were stained using monoclonal antibodies to cytokeratin (Chapter 2, section 2.3.3.)

5.2.4 Cell proliferation

Cell proliferation in normal fibroblasts cells from all of the six sites, cells derived from exuberant granulation tissue and those from verrucous, nodular, fibroblastic and mixed sarcoid tumours was investigated (Chapter 2, section 2.3.4). All of the results were expressed as the mean ± standard error (SEM). The statistical significance of the results was assessed using Student's t-test and a p value of <0.05 was considered to be significant.

5.2.5 Morphological examination of cells

Photomicrographs were taken 48 hours after subculturing, see Chapter 2, section 2.3.5.

5.3 RESULTS

5.3.1 Morphology

Fibroblasts from the primary cultures showed different characteristic morphologies. The cells from normal tissue showed the typical spindle shaped morphology described by many authors (Plate 5.1a). Plate 5.1b shows the granulation tissue derived fibroblasts which had a spread out and a more cuboidal appearance. Cells from the fibroblastic mixed and verrucous type sarcoid tissue were large, spread cells with spiky edges, (Plate 5.1c) but the cells derived from nodular sarcoid tissue (Plate 5.1d) appeared, smaller and rounder.

In the case of the healing and non-healing limb wound (10026), the cells from the non-healing exuberant granulating end of the wound (Plate 5.2a) appeared to be large spread cells with spiky edges similar to the fibroblastic mixed and verrucous type of sarcoid-derived cell. These cells were about twice the size of the cells grown from tissue taken from the top healing part of the wound, which displayed the characteristic granulation tissue-derived cell morphology (Plate 5.2b). The fact that the cells from the non-healing part of the wound were much larger meant that they became contact inhibited much sooner than the other cells.

The morphology of the equine epithelial cells is displayed in plate 5.3a. It shows epithelial cells growing in an confluent sheet. They are regular and polygonal in shape with a clearly defined edge. Plate 5.3b displays the same cells positively stained with α-cytokeratin to establish that they indisputably are epithelial cells.

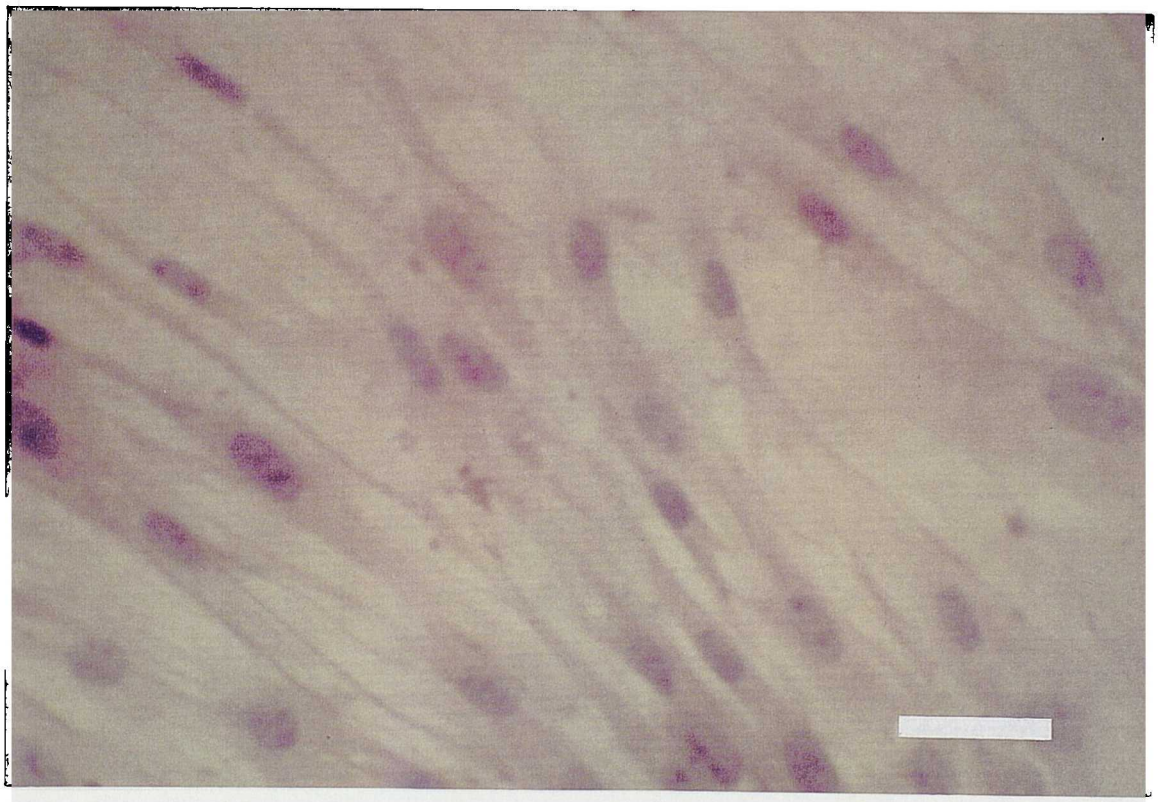


Plate 5.1a Phase contrast photomicrograph of cultured normal dermal fibroblasts. Bar: 100μm

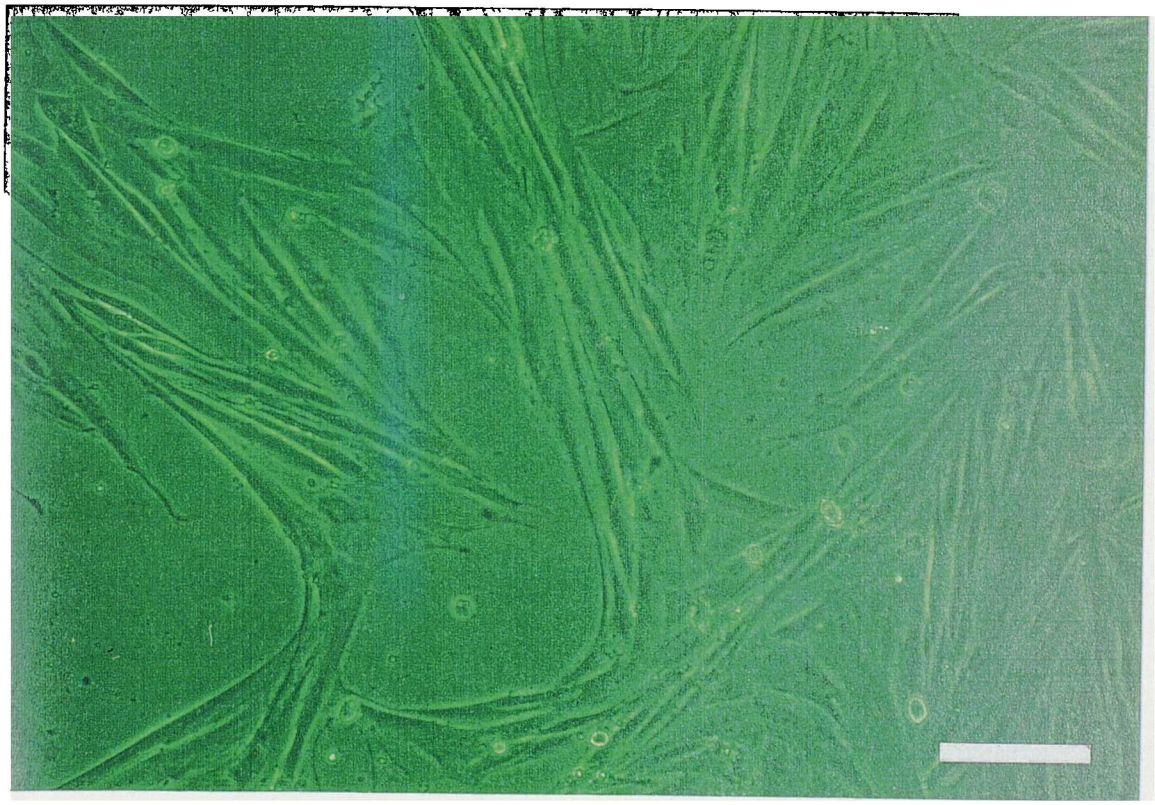


Plate 5.1b Phase contrast photomicrograph of granulation tissue-derived fibroblasts. Bar: 100μm

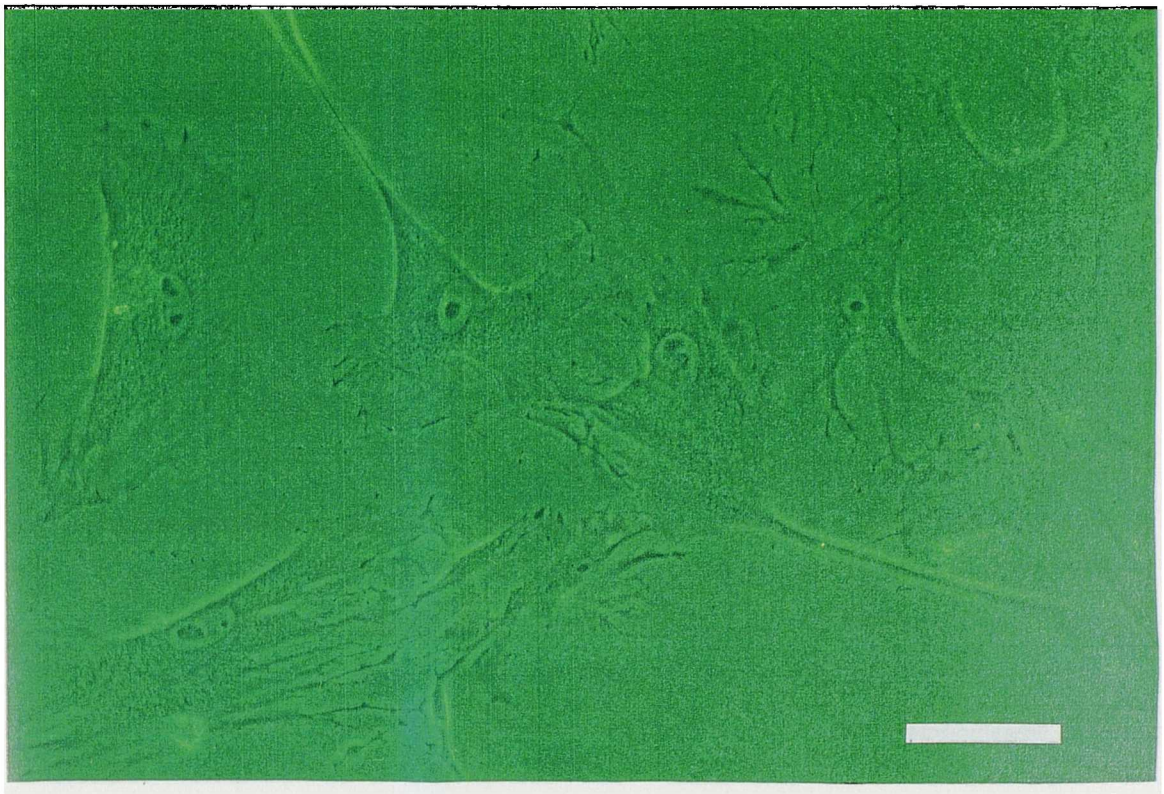


Plate 5.1c Phase contrast photomicrograph of fibroblastic sarcoid-derived fibroblasts. Bar: 100μm

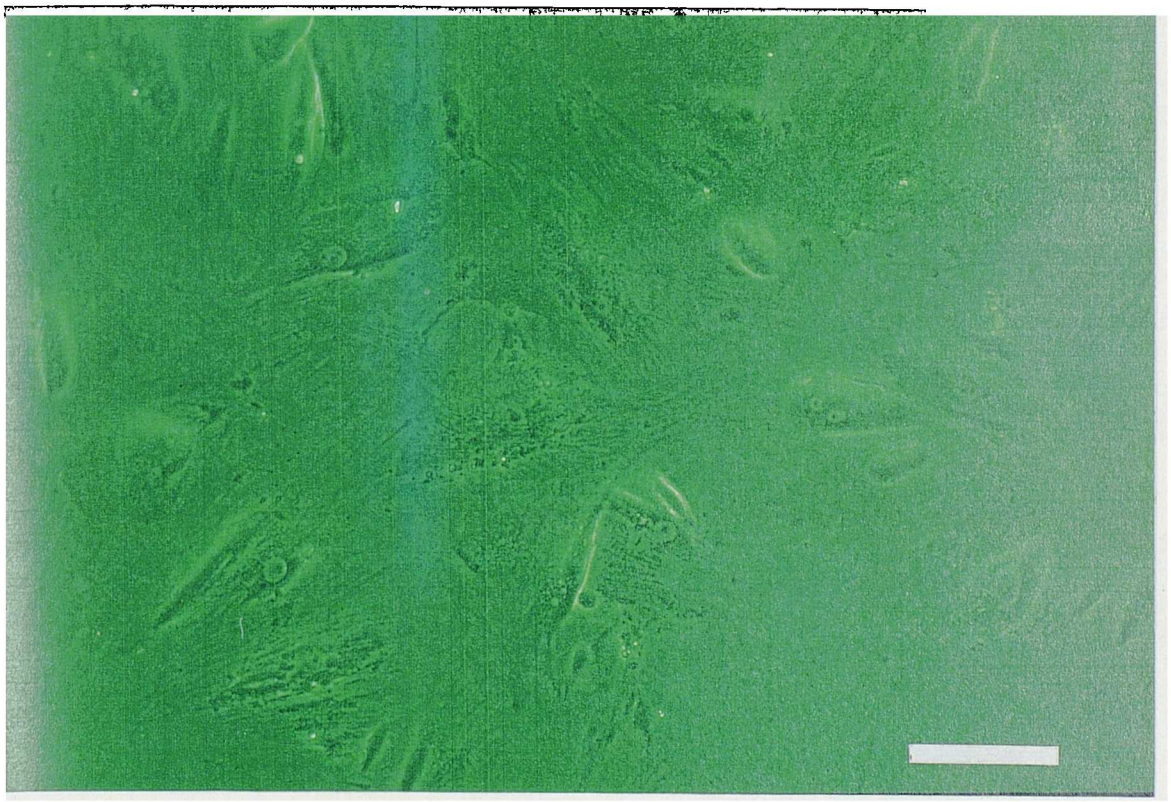


Plate 5.1d Phase contrast photomicrograph of nodular sarcoid-derived fibroblasts. Bar: 100μm

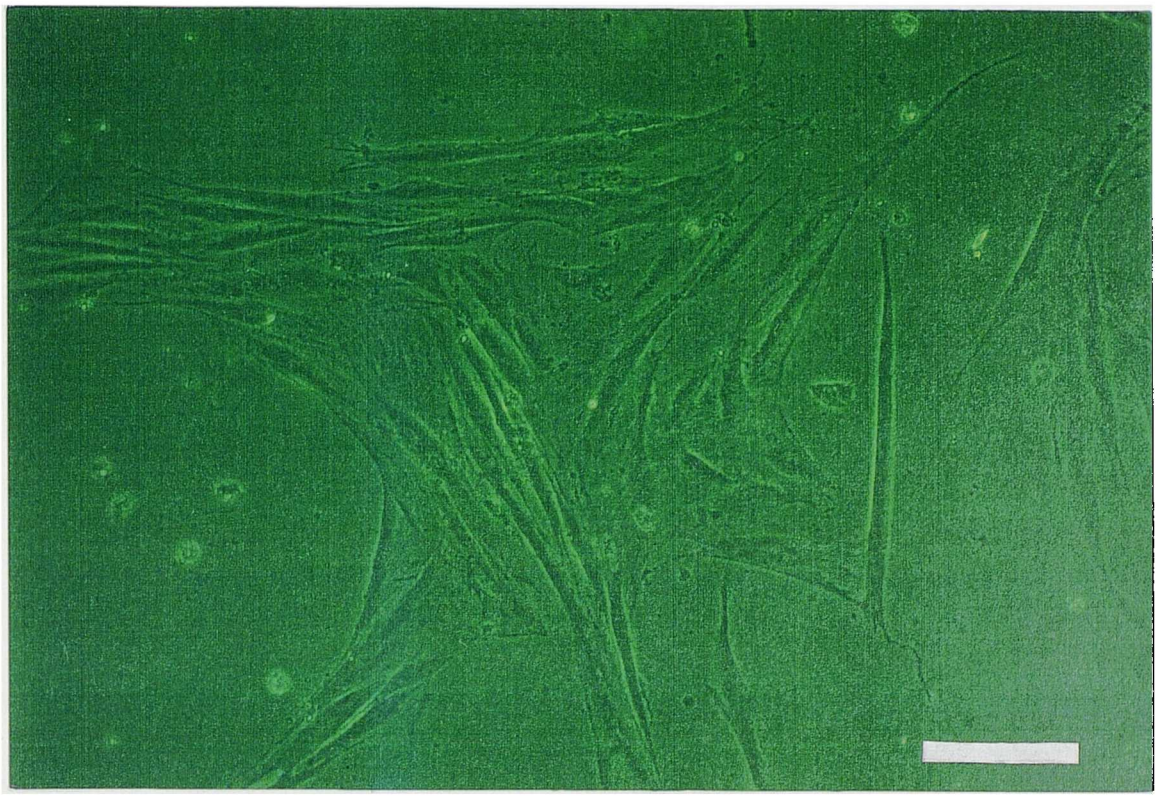


Plate 5.2a Phase contrast photomicrograph (Case 10026) fibroblasts derived from tissue extracted from the healing part of the wound. Bar 100μm

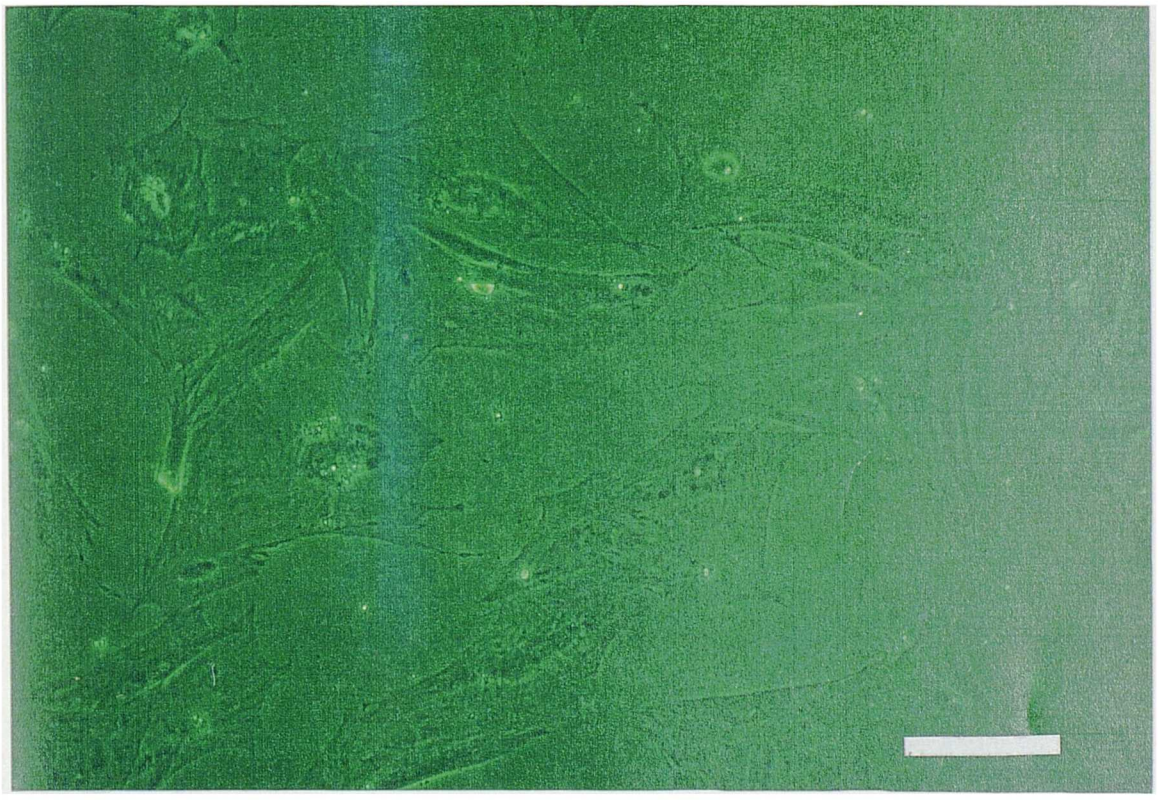


Plate 5.2b Phase contrast photomicrograph (Case 10026), fibroblasts derived from tissue extracted from the non-healing (granulating) part of the wound. Bar: 100μm

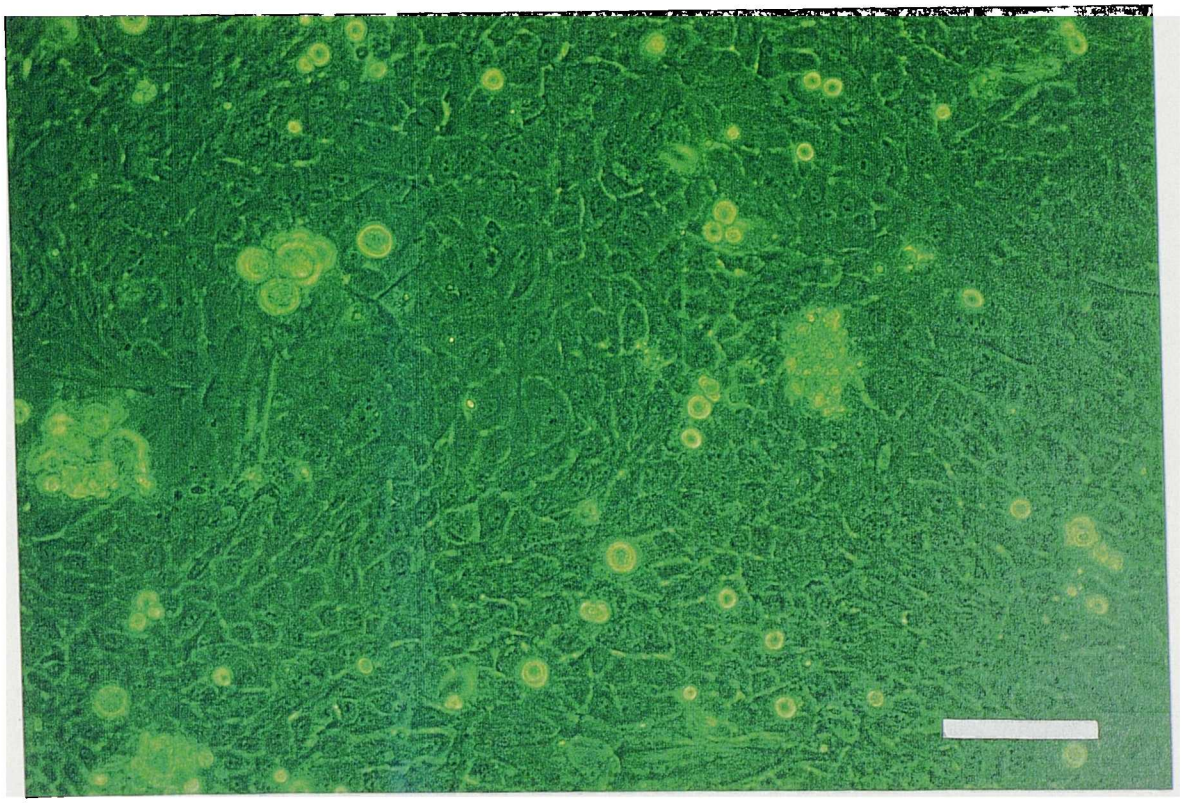


Plate 5.3a Phase contrast photomicrograph showing the morphology of epithelial cells. Bar: 100µm

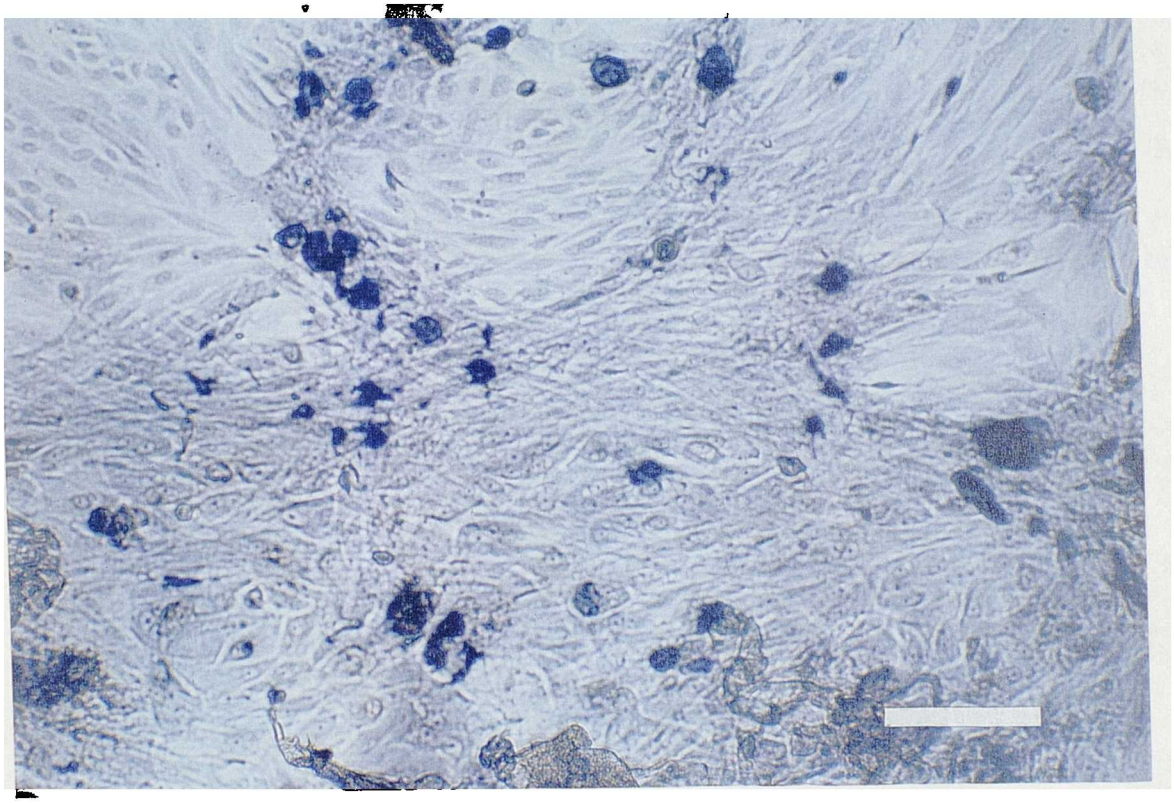


Plate 5.3b Phase contrast photomicrograph showing epithelial cells stained with α-cytokeratin. Bar: 100µm

5.3.2 Cell proliferation

Initial experiments on the normal dermal fibroblasts established that such cells proliferated in a dose dependant manner in response to the inclusion of foetal calf serum in the media and that the final saturation density was dependant on serum concentration (Figure 5.1).

Figure 5.2 demonstrates that the cells cultured from the normal dermis proliferated rapidly compared to the granulation tissue and sarcoid derived cells when treated with 2.5% FCS. A significant difference at day 5 was seen between the normal and the granulation tissue-derived cells ($P< 0.001$) and a difference between the normal and the sarcoid cells ($P<0.0001$).

At day 10, a similar trend occurred with a significant difference ($p<0.05$) between normal cells and granulation tissue-derived cells and a significant difference ($p<0.005$) between normal and sarcoid-derived cells.

The cell numbers for days 15 and 20 show, a significant difference ($p<0.01$) between the normal and the granulation tissue-derived cells and a significant difference ($p<0.001$) between the normal and the sarcoid-derived cells on both days. There was no difference between the granulation tissue and the sarcoid derived cells on any of the five counting days. A similar pattern is evident in Figure 5.3. Except that the proliferation rate is much lower with the serum free medium and the cell numbers of the granulation tissue and sarcoid-derived cells decreased over time whereas, the normal dermal fibroblasts continued to proliferate.

At days 5 and 10 the proliferation rate of the normal cells was significantly higher than the sarcoid and granulation tissue-derived cells ($p<0.02$).

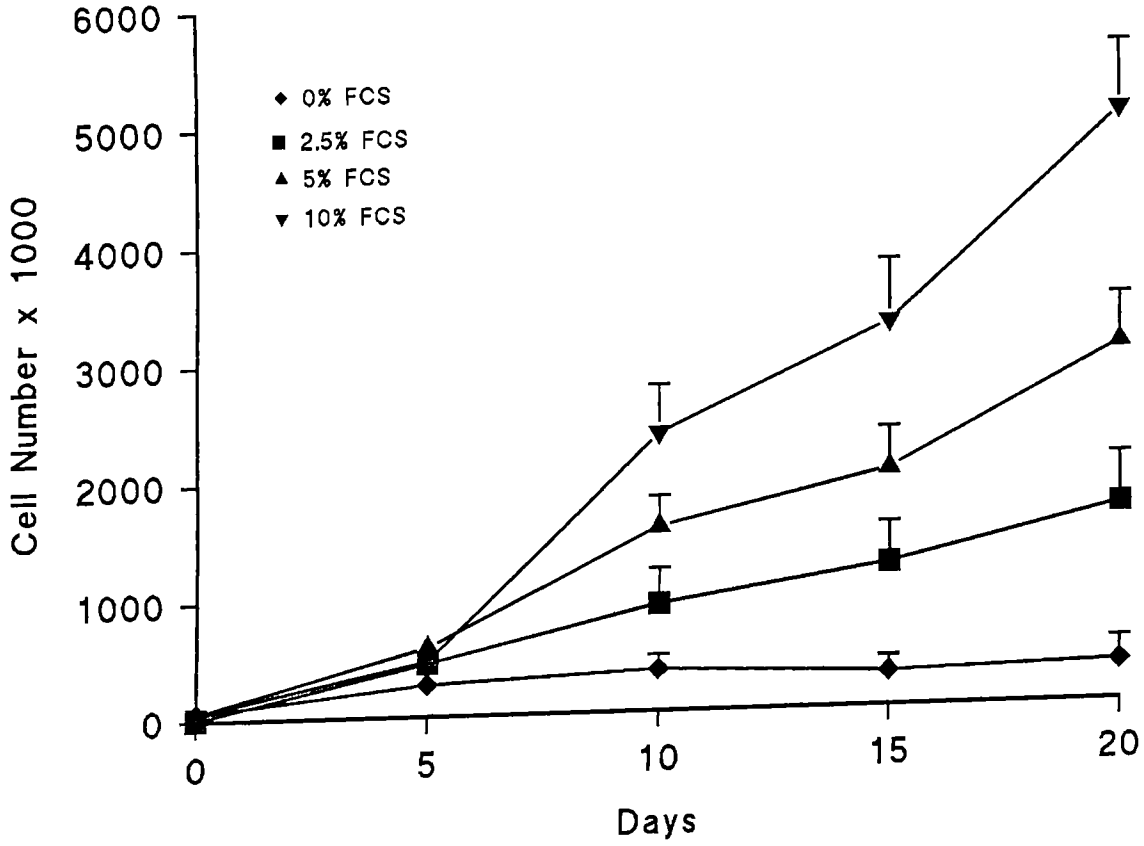


Figure 5.1 Effect of different concentrations of FCS on the proliferation of normal dermal fibroblasts. Normal dermal fibroblasts were grown in media containing 0%, 2.5%. 5% and 10% FCS for twenty days, cells were counted every five days (n=4).

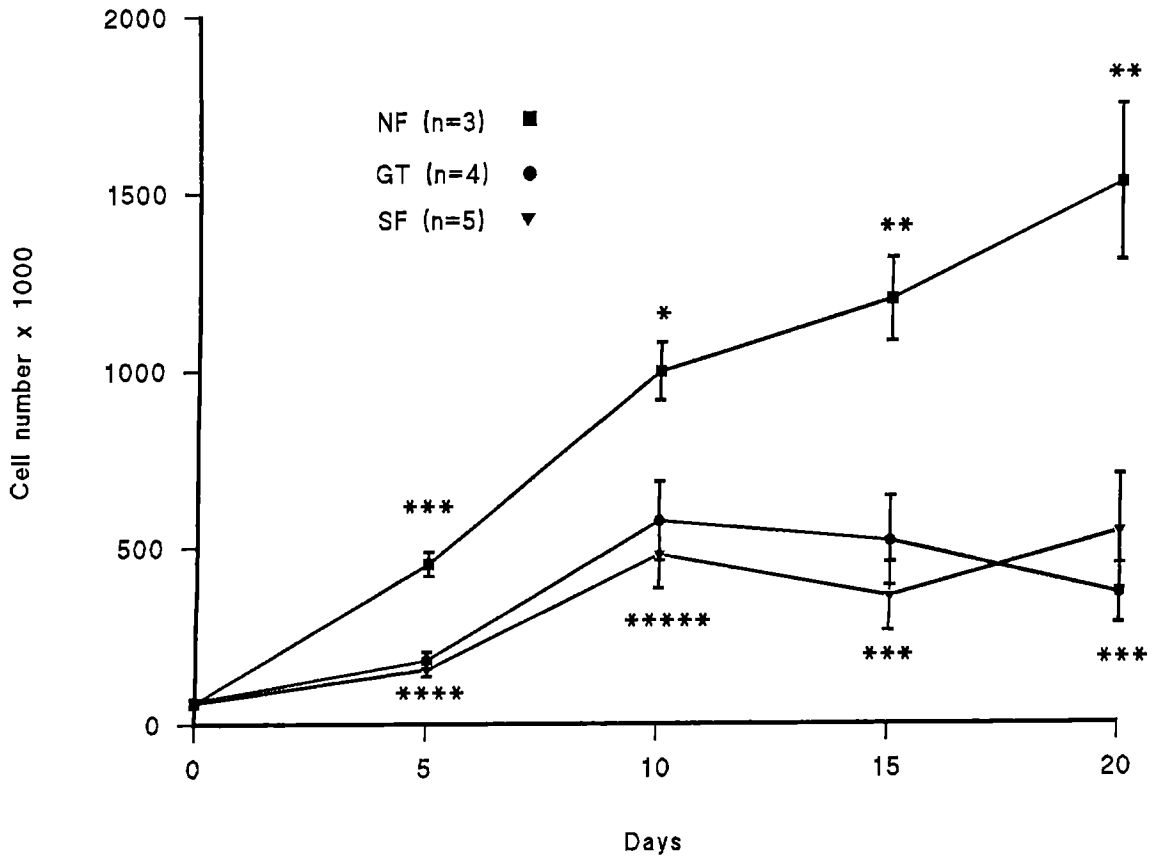


Figure 5.2 Growth rates of normal (NF), graulation (GT) and sarcoid-derived (SF) fibroblasts. Cells were grown for 20 days and counted every 5 days. Values significantly different in normal and granulation tissue-derived cells ***P<0.001; *P<0.05; **P<0.01. Values significantly different in normal and sarcoid tissue-derived cells ****P<0.0001; ***** P<0.005; ***P<0.001.

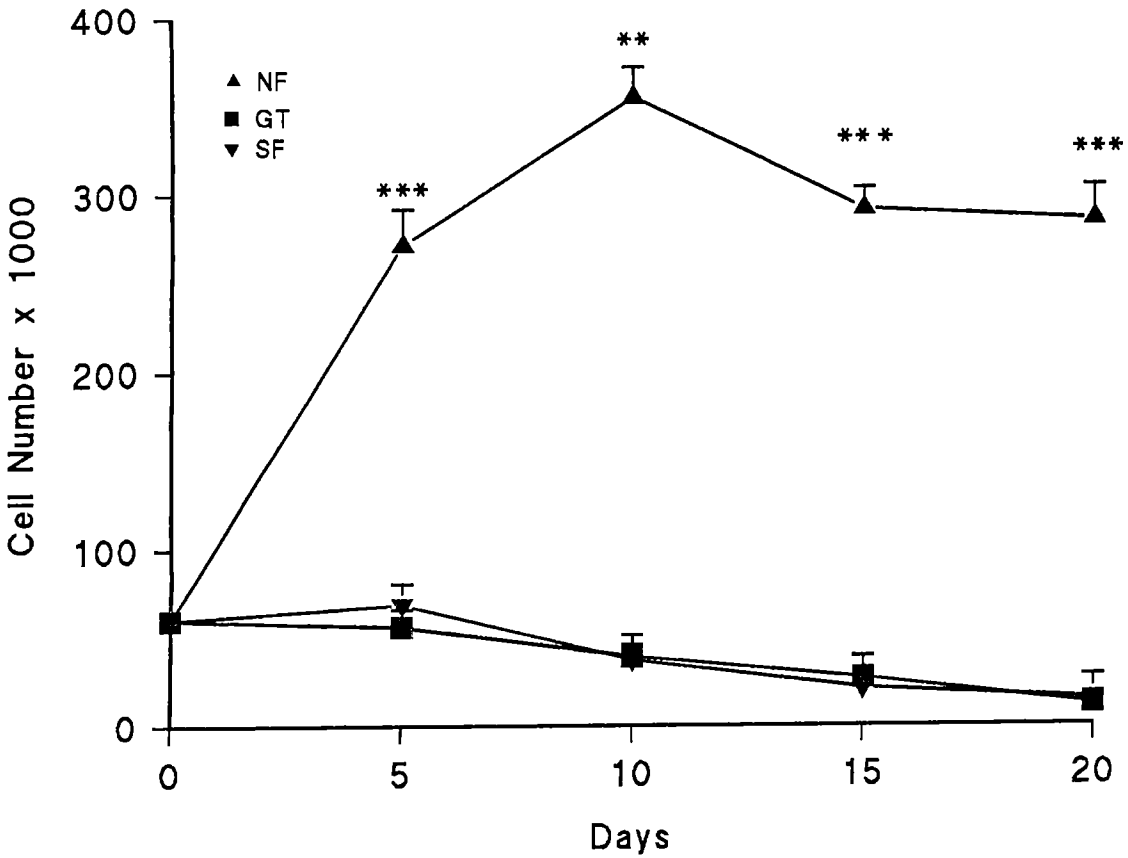


Figure 5.3 Effect of serum free media on the proliferation of normal, granulation and sarcoid-derived fibroblasts. Normal (NF) fibroblasts (n=3), granulation tissue (GT) derived fibroblasts (n=4) and sarcoid-derived (SF) fibroblasts (n=5) were grown in serum free media for 20 days and counted every five days. Values significantly different to normal and granulation and sarcoid-derived cells, * $P<0.02$; ** $P<0.005$; *** $P<0.001$.

On days 15 and 20 the growth rates for the normal cells were significantly higher than the granulation tissue and sarcoid derived cells ($p<0.005$) and ($p<0.001$) respectively. There was no significant difference between the sarcoid-derived and the granulation tissue-derived cells on any of the counting days.

Further proliferation studies were carried out comparing the six different sites in three different horses as described in the methods section. Normal dermal fibroblasts were taken from six sites of three individual horses and grown in 2.5% FCS for 20 days and counted every five days (Figure 5.4).

At day 5 cell proliferation in the limb cells was significantly lower ($p<0.001$) than in the cells from the groin and axilla. Also, a lower proliferation rate was seen in the limb when compared to the abdomen ($p<0.01$) and between the limb and thigh ($p<0.005$). There was however, no significant difference in growth rates between the limb and the eyelid. The proliferation rate in the limb was significantly higher ($p<0.0001$) than in the groin, axilla, eyelid and thigh on day 10.

By day 15 and 20, the proliferation rate of the limb cells was significantly higher ($p<0.0001$) than the groin, axilla, eyelid and thigh cells. There was however, a smaller increase ($p<0.001$) between the limb and the abdomen cells.

The above study was carried out in the same way except under serum free conditions. As in previous studies the cell numbers were greatly reduced (Figure 5.5), however similar patterns emerged as in the previous experiment. The limb cells displayed a significantly higher proliferation rate ($p<0.0001$) than the groin, axilla and eyelid cells at day 5. A significantly higher difference ($p<0.002$) was observed between the limb and the abdomen and ($p<0.001$) between the limb and the thigh.

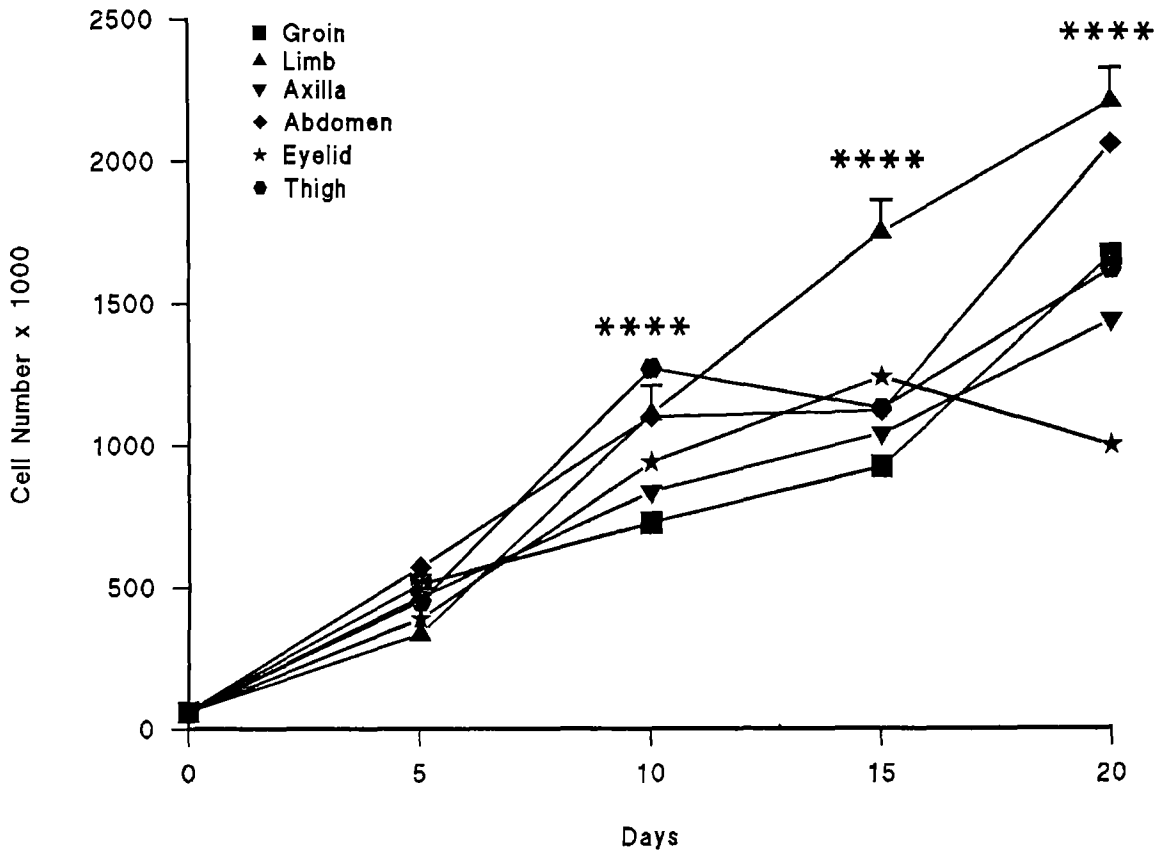


Figure 5.4 Proliferation study of normal dermal fibroblasts from six different sites in the horse. Normal dermal fibroblasts were cultured from six different sites of the horse and grown in media containing 2.5%FCS for 20 days and the cells were counted every 5 days (n=3). Values significantly different to limb and groin, axilla, eyelid **** $P<0.001$.

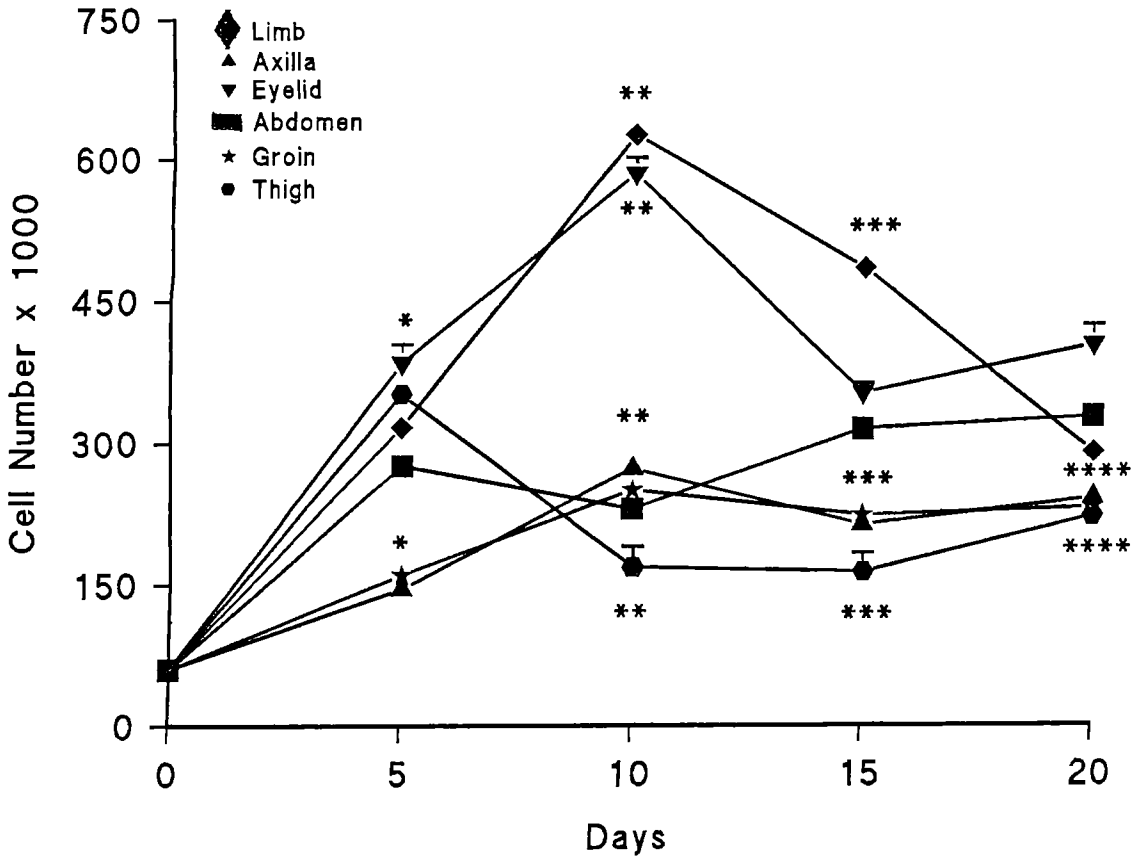


Figure 5.5 Proliferation study of normal fibroblasts cultured from six sites of the horse. Normal dermal fibroblasts were grown in serum free medium for 20 days and counted every five days (n=3). Values differed significantly to cells from the limb and the axilla, eyelid, and groin * $P<0.001$; day 5. Values differed significantly to cells from the limb and the axilla, eyelid, abodmen and thigh ** $P<0.001$; day10. Values differed significantly to cells from the limb and the axilla, abdomen, groin and thigh *** $P<0.001$ day 15. Values differed significantly from the limb and the axilla and thigh **** $P<0.001$ day 20.

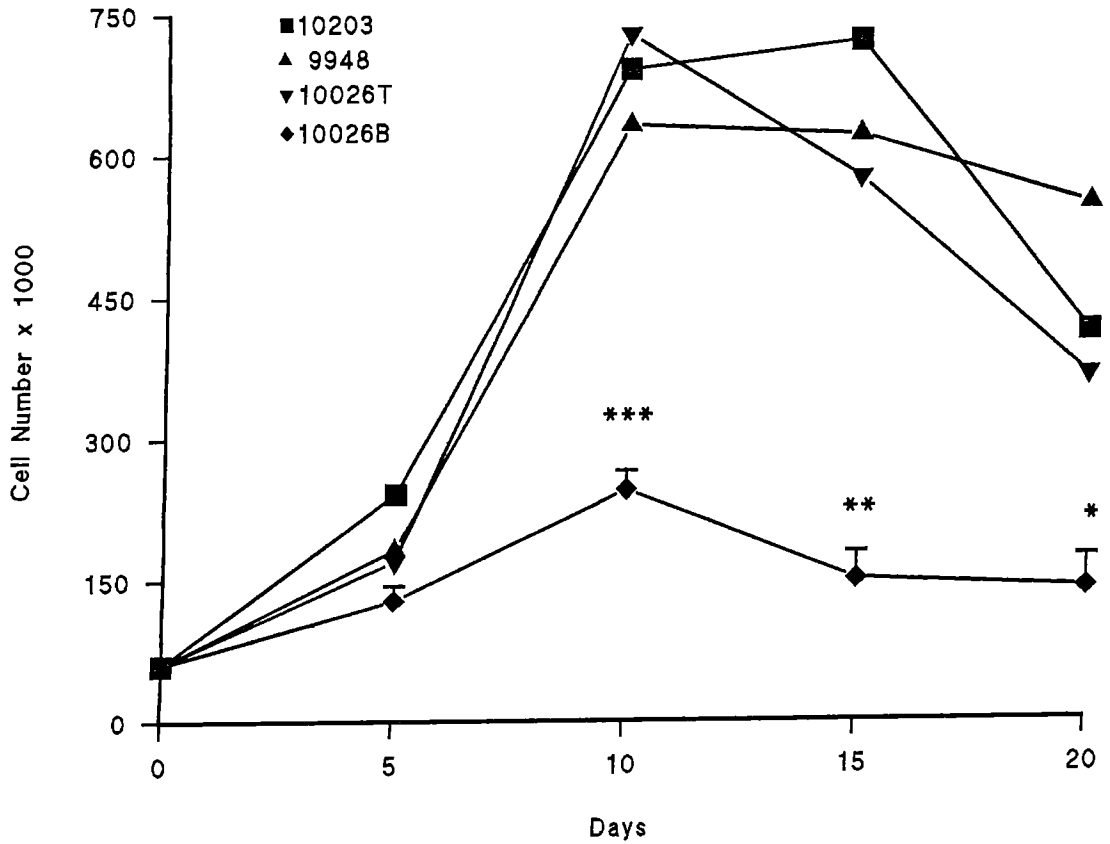


Figure 5.6 Study of cell proliferation of dermal fibroblasts from granulating wounds. Equine dermal fibroblasts from granulating wounds were grown for 20 days and counted every 5 days (n=3). Values significantly different to 10026B (cells cultured from the non-healing part of the wound) and other granulation tissue-derived cells. *** $P<0.005$ day 10; ** $P<0.01$ day 15 and * $P<0.05$ day 20.

The results for day 10 showed a significantly higher proliferation rate ($p<0.0001$) in the limb compared to the axilla, eyelid, abdomen and thigh and a significant difference ($p<0.001$) between the limb and the groin.

The limb derived fibroblasts were continuing to proliferate at a higher rate than the axilla, abdomen, groin and thigh cells ($p<0.0001$) on day 15. The difference however, was significantly lower ($p<0.001$) when compared to the eyelid.

On the final day of the study (day 20), the limb cells were continuing the same trend where they were significantly higher ($p<0.0001$) than the axilla and thigh cells. There was a slightly lower difference between the limb and the eyelid and groin cells ($p<0.001$) and an even lower difference between the limb and the abdomen cells ($p<0.05$). Studies were carried out to establish if there were any differences in the growth rates of exuberant granulation tissue-derived cells from different horses. Three cases were sampled, one of which was case 10026 which had the healing and non-healing wound. Figure 5.6 shows the results from this study, 10026T being cells cultured from tissue from the healing part of the wound and 10026B being cells cultured from tissue from the non-healing area. At day 5, there was no significant difference between cells cultured from the bottom of the wound (10026), compared to the cells from the healing part of the wound and the other granulation tissue-derived cells. There was however, a significant increase in cell proliferation of cells cultured from the granulation tissue other than that from the non-healing part of the wound on the other counting days these were: day 10, ($p<0.005$), day 15, ($p<0.01$) and day 20 ($p<0.05$). A similar pattern was seen when the experiment was carried out under serum free conditions but the cell numbers were greatly reduced.

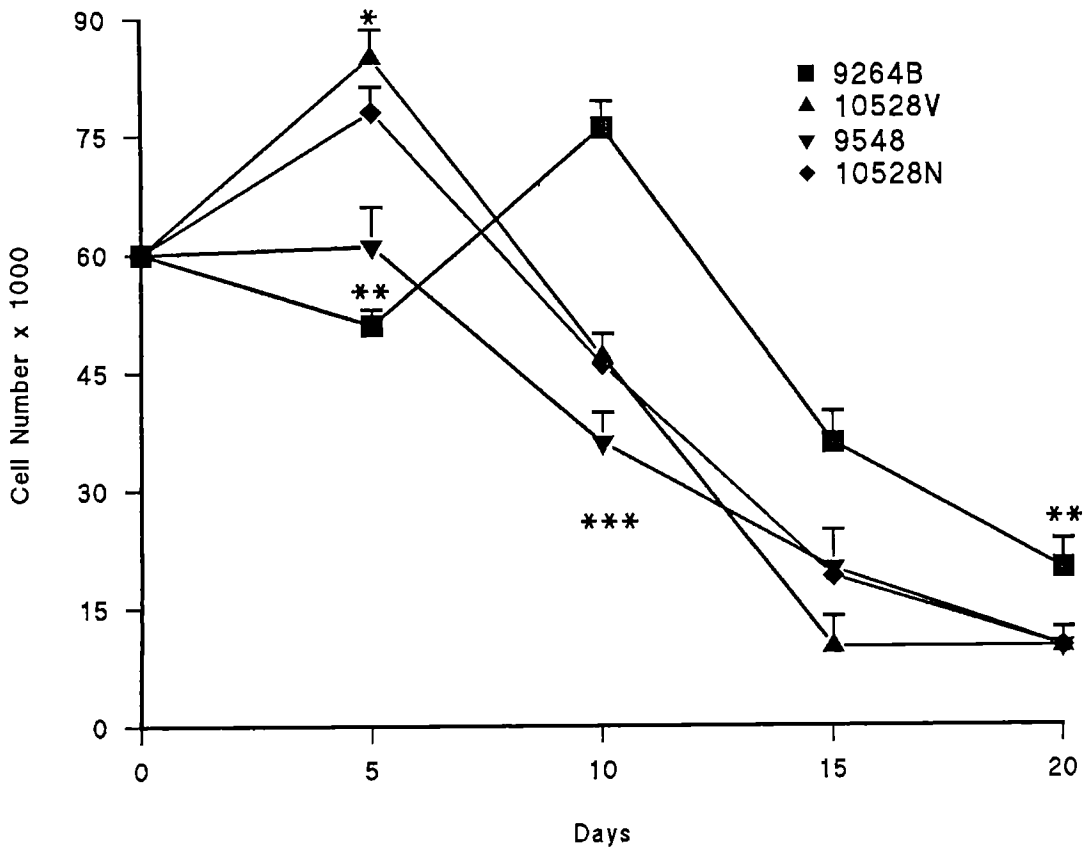


Figure 5.7 Study of cell proliferation of sarcoid-derived fibroblasts in serum free media. Sarcoid-derived fibroblasts were grown in serum free media for 20 days and counted every 5 days (n=3). Values differed significantly to 10528V and 10528N *P<0.005 day 5. Values differed significantly to 9264B (fibroblastic) and 10528 (verrucous and nodular) ** P<0.001 day 5; *** P<0.0001 day 10; ** P<0.001 day 20.

Proliferation studies were carried out to establish if there was any difference in proliferation between the different types of sarcoid-derived cells (Figure 5.7). In this study cells were grown under serum free conditions it is evident that the cell numbers are declining over time in all the cell types.

There was no significant difference between the verrucous and nodular sarcoid-derived cells except on day 5, when the verrucous cells had a significantly higher cell number than the nodular cells ($p<0.005$), case number 10528. It is evident that at day 5, 9264b (fibroblastic) cells had a significantly higher cell number ($p<0.001$), than the verrucous and nodular sarcoid cells and a difference ($p<0.002$) between the fibroblastic and the mixed (9548) sarcoid-derived cells. The results from day 10, reveal a significantly lower growth rate ($p<0.0001$) in the verrucous and nodular sarcoid-derived cells when compared to the fibroblastic cells. A smaller lower difference ($p<0.001$) was seen between the fibroblastic and the mixed sarcoid-type cells.

At day 15, the fibroblastic sarcoid-type cells (9264b) had a significantly higher proliferatiion rate than the other cell types ($p<0.001$). The proliferation rate of these cells was still higher than the other cells at day 20, with a significant difference ($p<0.001$) from the verrucous, ($p<0.05$) from the mixed and ($p<0.005$) from the nodular sarcoid cells. A similar pattern was seen in cells cultured in media containing 2.5% FCS.

Proliferation studies with epithelial cell cultures revealed the same trends that were seen with the normal dermal fibroblasts.

In subsequent experiments, the inclusion of 2.5% FCS was considered to be the lowest concentration that the cells could tolerate, since lower concentrations resulted in poor establishment and maintenance of cells.

5.4 DISCUSSION

To elucidate the *in vivo* mechanism of action of an agent, such as a growth factor, cell culture is a powerful tool if careful consideration of culture conditions is undertaken. It is likely that the cells isolated from their physiological environment acquire replicative and metabolic properties different from the *in vivo* situation. Therefore in the present study, early passage cells were used to attempt to preserve the physiological condition that exist *in vivo* as closely as possible. Also, cells only in the actively growing stage were used which may have reflected the state of cells growing in the wound environment.

This study demonstrated a difference in cell morphology and growth rate characteristics between normal fibroblastic cells, sarcoid-derived and granulation tissue-derived cells. The slow growth rates of granulation tissue and sarcoid tissue-derived cells could be due to cell to earlier cell contact inhibition occurring as cells are much larger and therefore the confluence level was achieved much sooner and with a lower number of cells. The morphology of the sarcoid and granulation tissue-derived cells differed from normal fibroblasts and it has been suggested that histopathological confirmation of the tissue types is necessary to distinguish sarcoid tissue from granulation tissue (Genetzky *et al.*, 1983; Tarwid *et al.*, 1985; Adams *et al.*, 1988). This could explain why the growth rates of these cells are so similar under normal conditions. Exuberant granulation tissue on the lower limbs of horses grows rapidly and may reach vast quantities (Fretz *et al.*, 1983; Jacobs *et al.*, 1984; Bertone *et al.*, 1985; Bertone, 1988; Lindsay, 1988), and we hypothesized that the growth rates of these granulation tissue-derived cells might have been much higher than the growth rates of the normal cells. However, *in vivo* granulation tissue is a highly vascular, resistant tissue which

suggests that the slower growth rates *in vitro* may be due to a lack of nutrients or growth factors which may be present in naturally occuring granulation tissue *in vivo*.

Proliferation studies of the normal fibroblasts comparing the six sites demonstrated that the cells cultured from the limb had a higher proliferation rate than the cells cultured from some of the other sites. However, the proliferation rate seemed to increase over time in both culture conditions. There were also similar differences between the cells from the other sites. Therefore, no definite conclusions can be drawn from this data. However, it suggests that the problem in equine wound healing is not related with any difference in proliferation rates of the dermal fibroblasts from the different sites. The difficulty in lower limb wound repair may be due to the anatomical location where increased movement, increased skin tension, reduced vascularity, thinness of skin, higher susceptibility to infection, reduced scope for wound contraction and lack of appreciable underlying muscle tissue (Bertone 1989a,b; Britton 1970, Jacobs *et al.*, 1984) may play a role. Collagen and growth factor production may also have an important role to play in equine wound healing problems.

In case 10026 the morphology of the cells grown from tissue taken from the top (healing part) of the wound were similar to the granulation tissue-derived cells but the cells grown from the bottom (non-healing) part of the wound were more similar to fibroblastic, mixed and verrucous type sarcoid-derived cells. These results suggest that the lower cell proliferation rate in the cells derived from the non-healing part of the wound could be due to the fact that these cells were much larger than the other granulation tissue cells, similar in morphology to the sarcoid cells and tend to proliferate at a similar rate to sarcoid cells. Therefore, contact inhibition would be activated, due to

the enlarged nature of the cells and the cell numbers would be reduced. This could possibly mean that this horse may develop sarcoids in the area of the non-healing, granulating wound in the future, although to date (2 years after discharge), there has been no sarcoid development and the wound healed well following grafting.

It has been demonstrated that there is a difference in the proliferation rates between the different sarcoid cell types, which could explain why they have different morphological and clinical characteristics (Knottenbelt *et al.*, 1995; Knottenbelt & Pascoe, 1994).

Although, initially the epithelial cells were difficult to culture, when the growing conditions were optimised it was possible to establish healthy differentiating cells which were pure in culture. It is hoped that it will be possible in the future to establish cultured epithelial cells on sheets of synthetic skin. These could be applied to non-healing wounds in an attempt to avoid the trauma and cost of skin grafting.

It is evident from all of the above studies that serum plays an important role in the culturing of equine cells. The slow growth of cells cultured in serum free conditions suggests that they require the growth factors present in serum in order to proliferate successfully. This is not surprising as serum is composed of a number of uncharacterised chemoattractants. The greater the concentration of serum the greater the increase in the cellular population. The viability of the granulation tissue-derived cells after cryopreservation was very poor and it was necessary to use only primary cultures for our experiments. The normal and the sarcoid-derived cells however, were still viable after cryopreservation. This could be due to the fact that the granulation tissue-derived cells may require higher concentrations of serum in the cryopreserving medium than the

normal and sarcoid-derived cells. In this chapter it was established that serum plays an important role in the maintainence and culture of dermal cells however, in Chapter Six, the role of growth factors will be discussed.

CHAPTER SIX

The effects of growth factors on cell proliferation and morphology in granulation tissue, sarcoid-derived and normal dermal fibroblasts

6.1 INTRODUCTION

Previous investigations (Chapter 5), indicated that there was a significant difference between the proliferation rates of fibroblast cells cultured from the limb and other sites in the equine body and fibroblast cells cultured from granulation tissue from healing and non-healing wounds. We therefore decided to investigate the response of these cells and fibroblast cells of sarcoid origin to cellular growth factors. Under normal circumstances the healing process is achieved by wound debridement, production of granulation tissue, reduction of the wound size by contraction and permanent surface protection by epithelial migration. However, this is not always the case in horses where wound healing is commonly delayed and is more complicated than in other species (Bertone, 1989a; Jacobs *et al.*, 1984). The formation of exuberant granulation tissue on the lower limbs of the horse often halts or delays wound healing. It is not known if growth factors play a role in this process or not as there has been no research conducted in this area of equine wound healing.

Likewise, research in this area on the equine sarcoid has not been published. Since sarcoids are the most common cutaneous tumours in horses and are the cause of a large number of horses being euthanised each year it was considered that the

investigation of the role of growth factors in sarcoid proliferation would be expedient. Sarcoids may occur singly or in clusters on any part of the body, but the head, ventral abdomen and limb are most affected (Jackson, 1936) and it is not uncommon for sarcoids to appear at the site of wounds (Knottenbelt and Pascoe, 1994).

Most growth factors are mitogenic for normal human dermal fibroblasts in culture (Kingsnorth & Slavin, 1991). Basic FGF is mitogenic and chemotatic for endothelial cells *in vitro*, suggesting a role in new vessel formation (angiogenesis) and granulation tissue formation (Terranova *et al.*, 1985). In this chapter normal dermal, granulation tissue and sarcoid-derived fibroblasts were cultured with several growth factors to try to assess the role of these growth factors in equine wound healing and sarcoid formation.

6.2 MATERIALS AND METHODS

6.2.1 Source of tissue and dermal cell culture

Dermal Tissue was obtained from horses described in Chapter 2, Table 2.2. Tissue samples were immediately transferred to a dish of Hank's balanced salt solution and cultured as described in Chapter 2, section 2.3.1.

6.2.2 Cell proliferation

Cultured cells from normal skin, granulation tissue and sarcoid lesions were plated onto 35 mm plastic dishes and their proliferation rates were assessed as described in Chapter 2, section 2.3.4. All of the results were expressed as the mean ± standard error (SEM). The statistical significance of the results was assessed using Student's t-test and a p value of <0.05 was considered to be significant.

6.2.3 Photomicroscopy

Photomicrographs were taken 48 hours after subculturing as described in Chapter 2, section 2.3.5.

6.3 RESULTS

6.3.1 Morphology

6.3.1.1 Effects of Growth Factors

Addition of growth factors to the medium had an effect upon the morphology of all types of cells (Table 6.1). In media containing TGFβ the cells underwent distinct

changes in morphology, becoming flattened and enlarged with an apparently increased cytoplasmic/nuclear ratio which was not quantified (Plate 6.1a). The normal and granulation tissue-derived fibroblasts (Plate 6.1b) adopted a thinner spindle shaped morphology with FGFb, whereas the sarcoid-derived cells produced much shorter compact spindles (Plate 6.1c). Granulation tissue-derived fibroblasts grown in media containing aFGF (Plate 6.1d) had irregular shaped cells but the sarcoid-derived cells transformed to larger flatter cells similar to the morphological changes that occurred with TGFβ (Plate 6.1e). The normal cell morphology remained the same. Media supplemented with EGF had little change on the morphology of the normal cells. However, the sarcoid cell morphology was altered to more flatter, spread and larger cells (Plate 6.1f) than the control cells. In contrast, EGF treated granulation tissue-derived cells altered their morphology to that similar to the morphology of the fibroblastic sarcoid cells, being large, expanded cells with spiky looking edges (Plate 6.1g). These morphological differences were seen each time the growth factors were added to the cultures. There appeared to be no alterations in these morphological changes as the cells neared confluence.

Table 6.1: Changes in cell morphology with the addition of growth factors

	UNTREATED	EGF	FGFa	FGFb	TGFβ
NORMAL FIBROBLASTS	spindle shaped characteristic morphology	spindle shaped (as untreated cells)	spindle shaped (as untreated cells)	thinner spindle shaped (plate 2b)	flattened enlarged (plate 2a)
GRANULATION TISSUE - DERIVED FIBROBLASTS	spread out cuboidal appearance (plate 1a)	large expanded cells with spiky edges (plate 2g)	irregular shaped cells (plate 2d)	thinner spindle shapes (plate 2a)	flattened enlarged cells (plate 2a)
SARCOID - DERIVED FIBROBLASTS	**fibroblastic, verrucose and mixed** large,spiky edges (plate 1b) **nodular** smaller, rounder (plate 1c)	flatter, spread out and larger cells (plate 2f)	large flatter cells (plate 2e)	shorter compact spindles (plate 2c)	flattened enlarg d cells (plate 2)

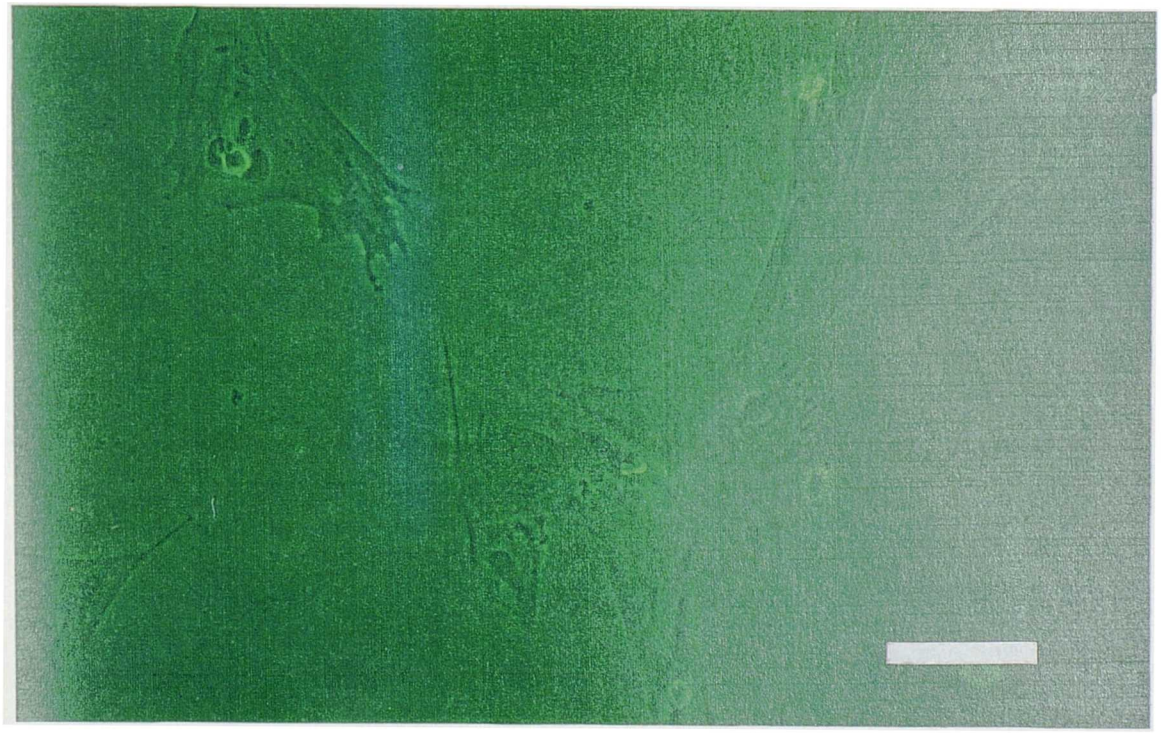


Plate 6.1a Phase contrast photomicrograph of cultured cells after treatm nt w'th TGFβ

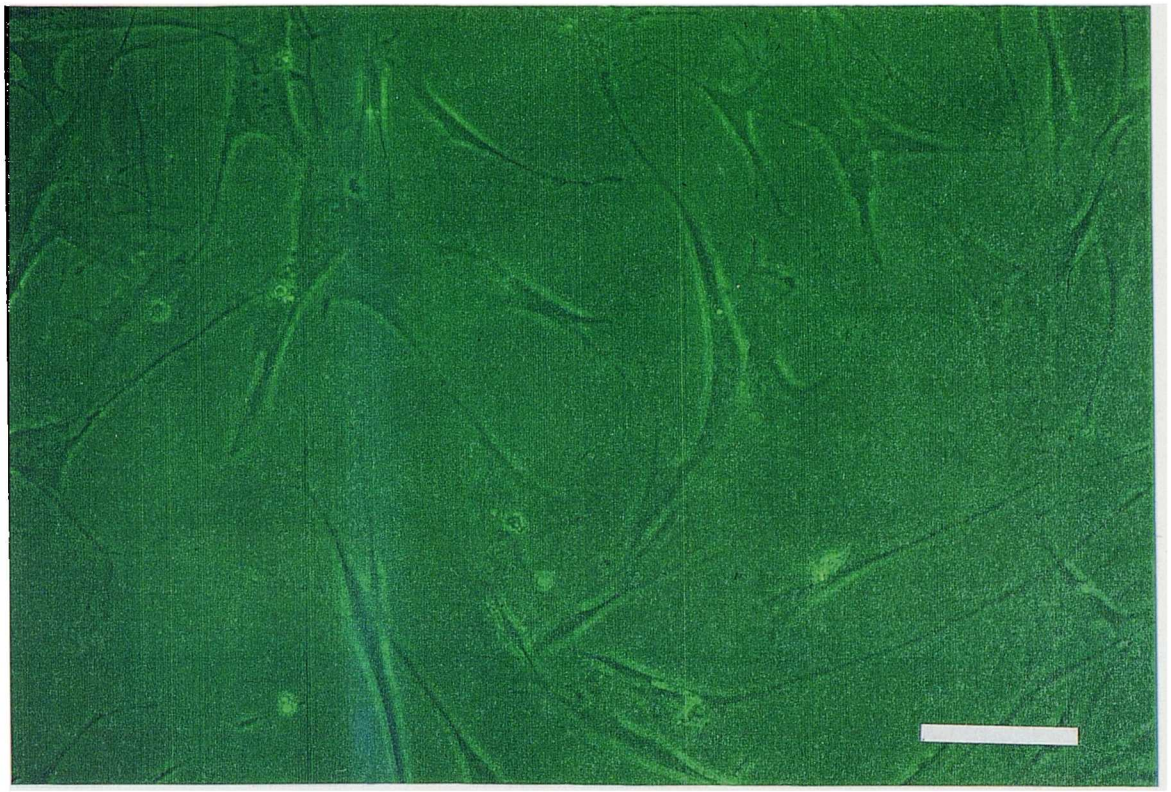


Plate 6.1b Phase contrast photomicrograph of normal and granulation tissue-derived cells after treatment with FGFb

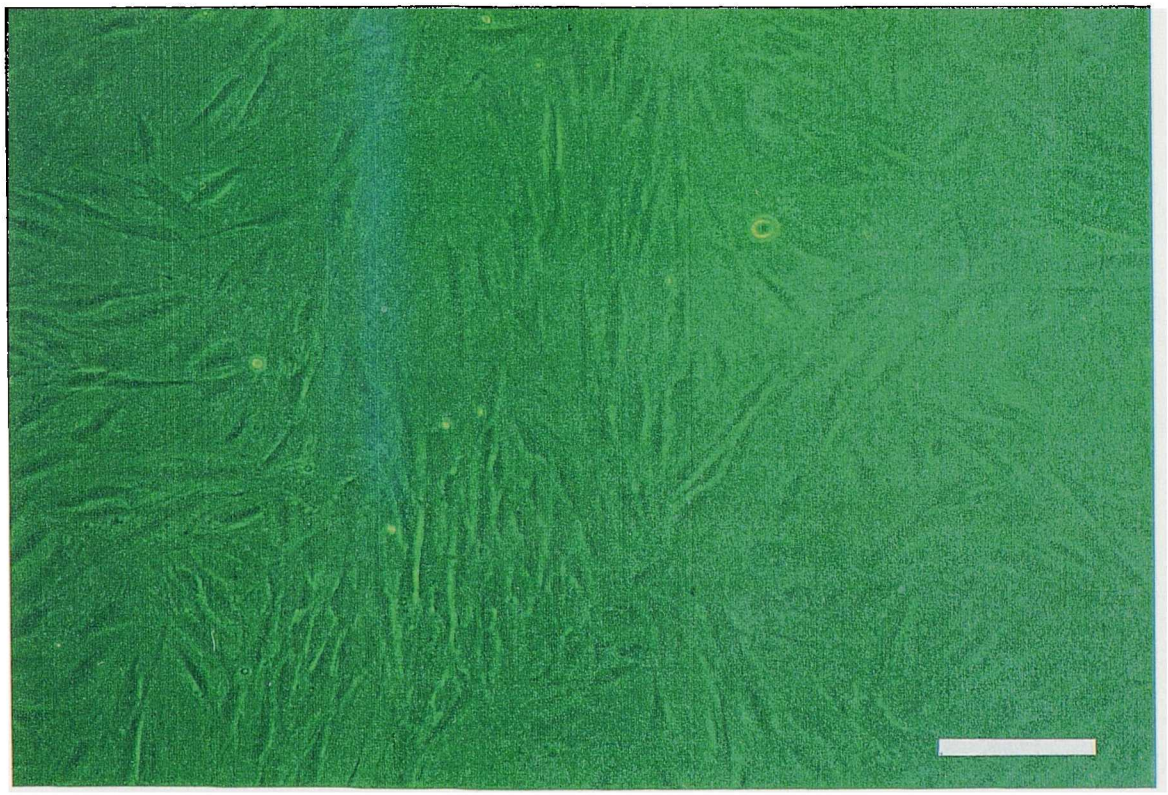


Plate 6.1c Phase contrast photomicrograph showing sarcoid-derived fibroblasts after treatment with FGFb

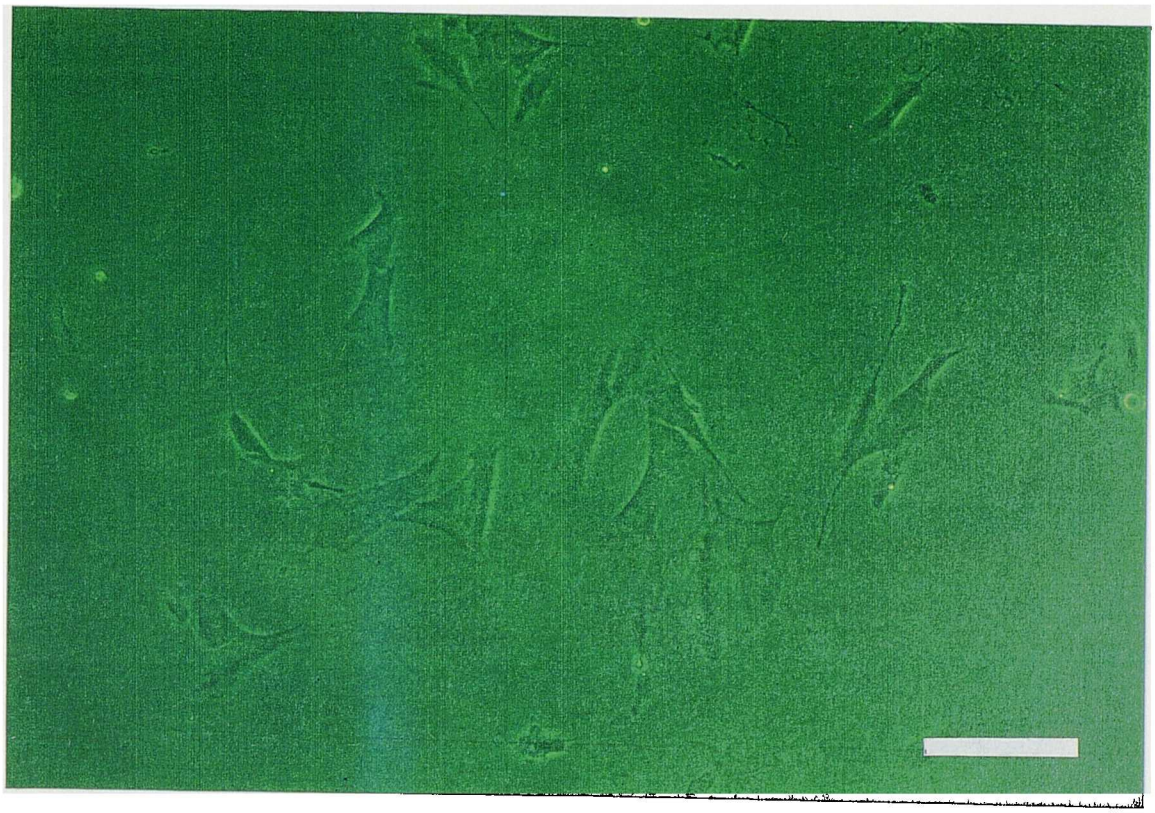


Plate 6.1d Phase contrast photomicrograph showing granulation tissue-derived fibroblasts after treatment with FGFa

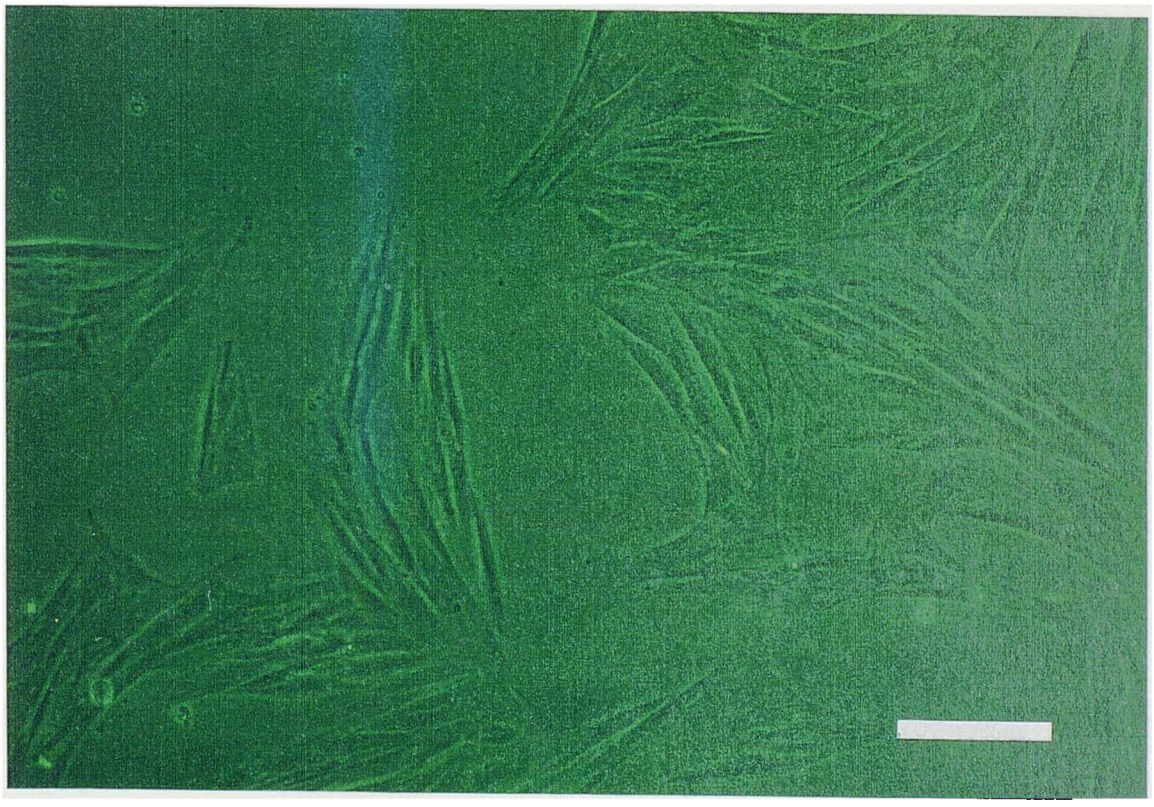


Plate 6.1e Phase contrast photomicrograph showing sarcoid-derived fibroblasts after treatment with FGFa

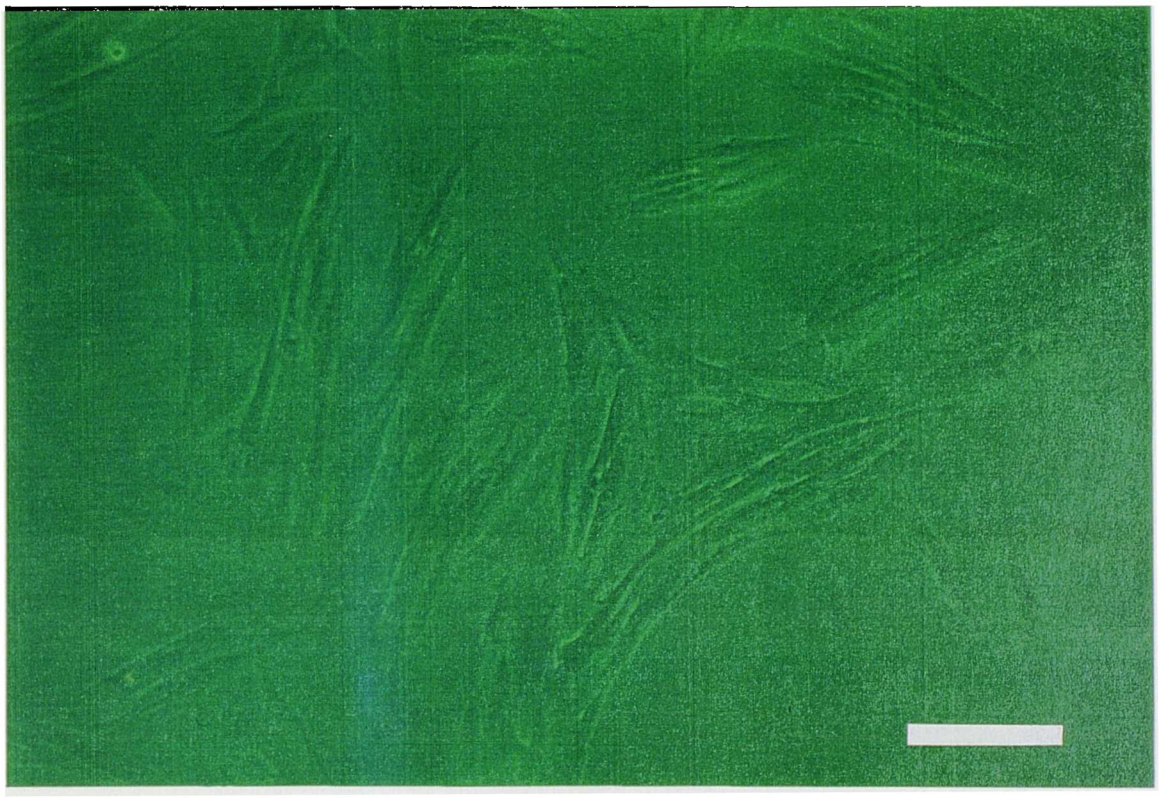


Plate 6.1f Phase contrast photomicrograph showing sarcoid-derived fibroblasts after treatment with EGF

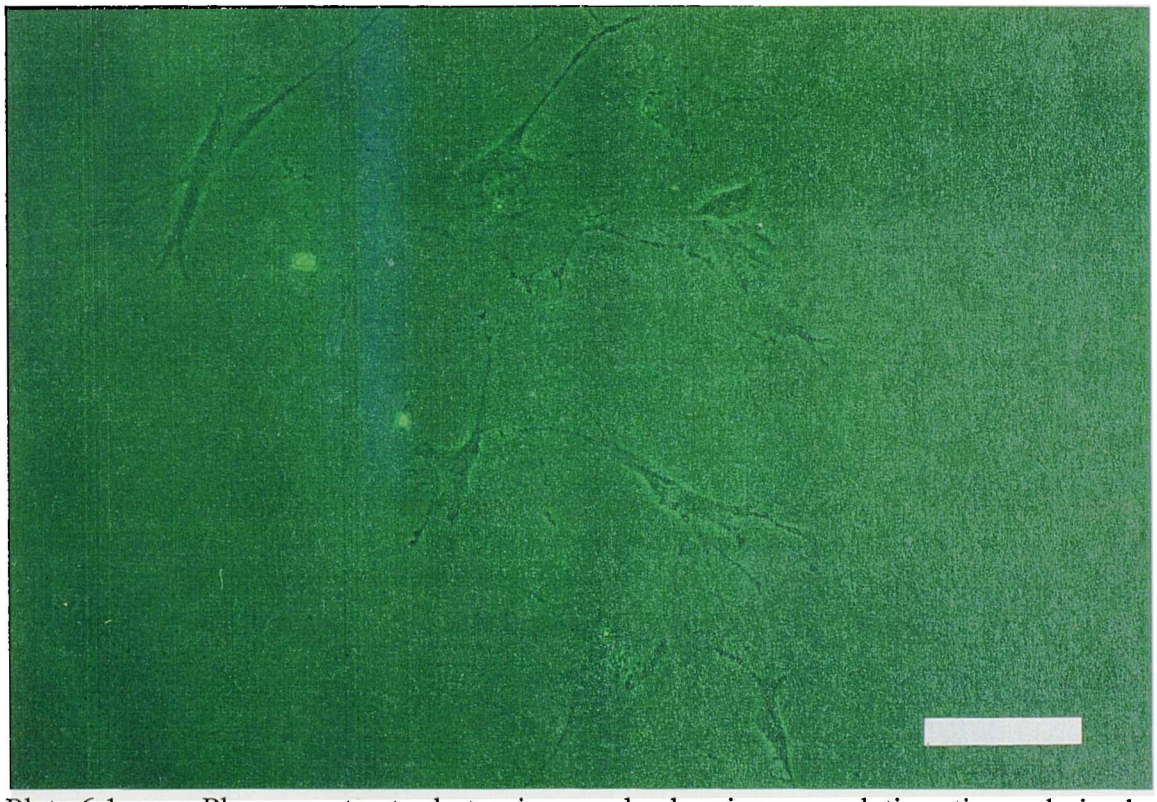


Plate 6.1g Phase contrast photomicrograph showing granulation tissue-derived fibroblasts after treatment with EGF

6.3.3 Growth rates

6.3.3.1 Supplementation with growth factors

A) Effect of EGF

Figure 6.1A shows the effect of EGF on the growth rates of the three cell types. On day 5, there was no significant difference between the normal cells and the other cells with EGF. However, the sarcoid cells had proliferated at a higher rate than the normal and the granulation tissue cells. The granulation tissue derived cells had the lowest proliferation rate.

At day 10, the normal cells showed a significantly higher proliferation rate ($p<0.005$) than the granulation tissue derived cells but no difference between the normal and sarcoid derived cells. There was also no significant difference between the sarcoid and granulation tissue derived cells. EGF had and inhibitory effect on the granulation tissue derived cells at day 15. The normal cells showed a greater response to EGF than the granulation tissue derived cells ($p<0.002$). Likewise the sarcoid cells showed a similar response with a significant difference ($p<0.05$) from the granulation tissue cells. On the final day of the experiment (day 20), there was a significant difference ($p<0.001$) in growth rates between the normal and granulation tissue cells treated with EGF. There was a greater stimulatory effect on normal cells with EGF than on the sarcoid tissue derived cells ($p<0.002$). A greater stimulatory effect was observed on sarcoid derived cells than on the granulation tissue derived cells ($p<0.05$) with the addition of EGF.

B) Effect of FGFa

The addition of FGFa, (Figure 6.1B) to the media of normal cells increased the cell numbers at day 10 but at a lower rate than cells treated with EGF. The sarcoid

derived cells followed a similar pattern. However, the addition of FGFa to the media of granulation tissue-derived cells appeared to slow down the proliferation rate. The normal cells had a significantly higher growth rate ($p<0.005$) than the granulation tissue -derived cells. At day 15, treatment with FGFa had stimulated the normal cells to proliferate but had the opposite effect on the granulation tissue derived cells ($p<0.0001$). At the end of the experiment (day 20), the growth rates of the normal cells was significantly higher ($p<0.001$) than the granulation tissue cells, treated with FGFa . There was a greater stimulatory effect on the normal cells with FGFa ($p<0.001$) than on the sarcoid derived cells.

C) Effect of FGFb

When the cells were stimulated with FGFb at day 10 (Figure 6.1C) there was a greater growth response than with FGFa; cell numbers increased with all cell types except the granulation tissue-derived cells which decreased in cell numbers. The normal cells showed a significantly higher cell replication rate ($P<0.02$) than the granulation tissue-derived cells.

D) Effect of TGFβ

TGFβ had a slight stimulatory effect at day 10, on all three cell types and there was a significant difference between the normal and granulation tissue cells ($p<0.05$).

TGFβ had a stimulatory effect on the granulation tissue derived cells at day 15 (Figure 6.1D), whereas the other growth factors had an inhibitory effect. The normal cells showed a greater response to EGF than the granulation tissue derived cells ($p<0.002$). Likewise the sarcoid cells showed a similar response with a significant difference ($p<0.05$) from the granulation tissue cells.

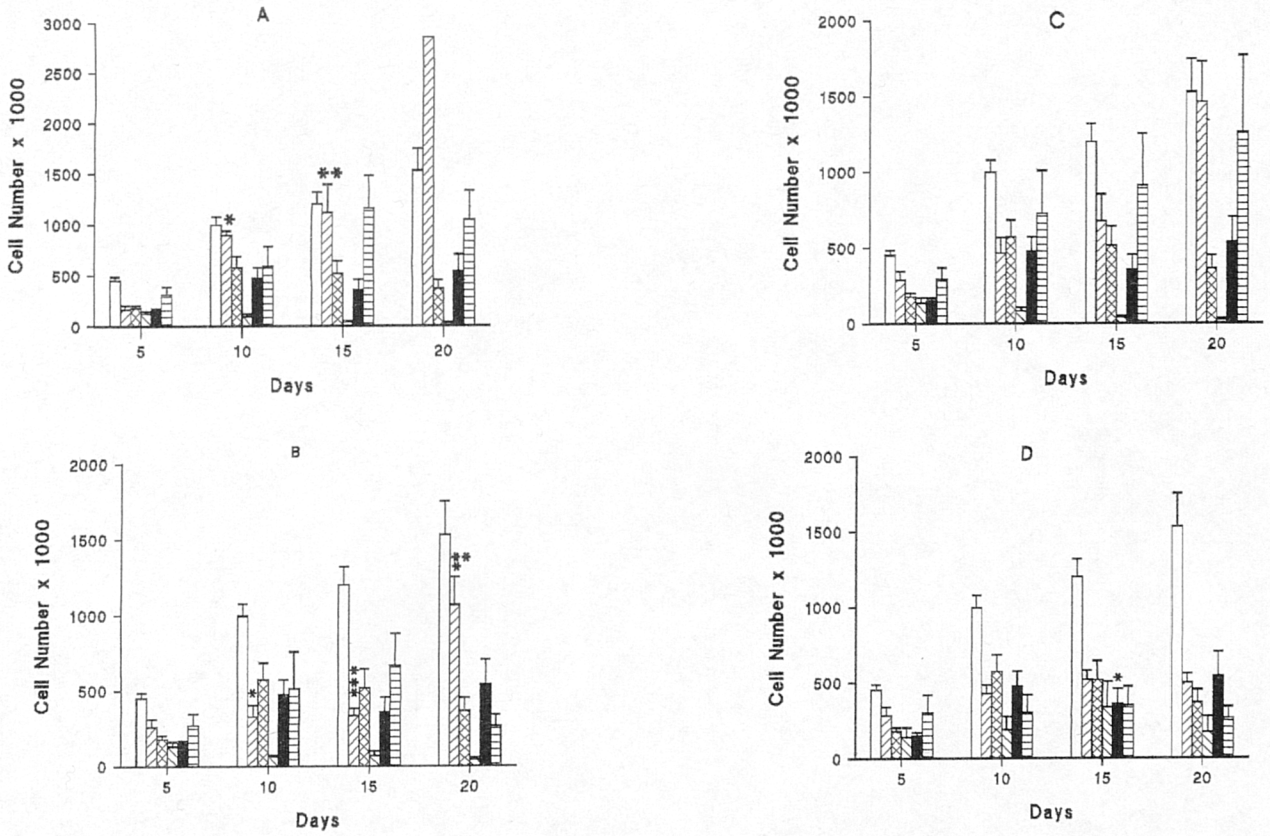


Figure 6.1 Growth rates of normal (n=3), granulation tissue (n=4) and sarcoid (n=5) fibroblasts treated with EGF, FGFa, FGFb at 10ng/ml or TGFβ 1ng/ml for 20 days and counted every 5 days. **A.** Effect of addition of EGF, **B.** FGFa, **C.** FGFb , **D.** TGF β. Untreated control cells (□); treated normal fibroblasts (▨); untreated granulation tissue-derived cells (▩); treated granulation tissue-derived cells (▧); untreated sarcoid-derived cells (■); treated sarcoid-derived cells (▤). Values differed significantly to normal cells treated with EGF and granulation tissue treated cells ** P<0.005 day10; *** P<0.002 day15 and **** P<0.001 day 20. With FGFa treatment values differed significantly to normal and granulation tissue-derived cells ** P<0.005 day 10; ***** P<0.0001day 15; ***** P<0.0001 day 20. Cells treated with FGFb differed significantly to normal treated and granulation tissue treated cells * P<0.02. Cells treated with TGFβ *** P<0.002 day 15.

In summary, treatment with FGFa stimulated normal cells to proliferate but had the opposite effect on the granulation tissue derived cells ($p<0.0001$). FGFb stimulated normal cells to grow at a higher rate than granulation tissue derived cells ($p<0.005$). On the final day of the experiment (day 20), there was a significant difference ($p<0.001$) in growth rates between the normal and granulation tissue cells (the normal cells having a greater proliferation rate than the granulation tissue cells), treated with EGF, FGFa, FGFb and a difference of ($p<0.05$) with the addition of TGFβ (Figure 2D). There was a greater stimulatory effect on normal cells with EGF than on sarcoid tissue derived cells ($p<0.002$). A similar effect was seen with FGFa ($p<0.001$) and TGFβ ($p<0.05$). A greater stimulatory effect was observed on sarcoid derived cells than on the granulation tissue derived cells ($p<0.05$) with the addition of EGF and FGFa.

There was no significant difference detected between the limb fibroblasts and fibroblasts derived from any of the other five sites with the addition of any of the growth factors. Likewise no difference was observed between the different types of sarcoids or granulation tissue taken from superficial, middle or deep sites within the wound with the addition of any of the growth factors.

6.4 DISCUSSION

All three cell types showed distinct morphological changes with the addition of TGFβ to the medium. This might be due to higher concentrations of these factors than would normally be found *in vivo.* A more likely explanation however, is that these cells needed to have contact with other extracellular matrix molecules e.g. collagen or fibronectin.

In the growth rate studies TGFβ inhibited the proliferation of the normal and sarcoid derived cells but had a lower inhibitory effect on the granulation tissue cells. TGFβ can increase the rate of cell proliferation in some fibroblast cell types via induction of a platelet derived growth factor (PDGF) - like protein (Loef *et al.*, 1986), but its predominant effect is to inhibit cell growth (Moses *et al.,* 1985). It is possible that the addition of TGFβ neutralising antibody would reverse the proliferative effect that TGFβ had on the granulation tissue-derived cells. Shah and colleagues (1992) found the application of TGFβ neutralising antibody at the time of wounding was essential to reduce active TGFβ levels, limit macrophage infiltration and further TGFβ release. Higher levels of endogenous TGFβ may be present in the granulation tissue than in the other tissues used for primary culture.

EGF was strongly stimulatory for normal cells with cell numbers steadily increasing over time. The same effect was seen with sarcoid cells but to a lesser extent. This could be due to sarcoid cells having fewer EGF receptors on their cell surfaces than normal cells. Normal human cells have approximately 20,000 EGF receptors per cell (Gill *et al.,* 1987). Increased proliferation is a common cellular response to EGF (Armelin, 1973; Cohen *et al.*, 1975) but EGF had an inhibitory effect on the granulation

tissue derived cells. The sarcoid and granulation tissue derived cells also showed altered morphology in the presence of EGF. Cohen (Cohen *et al.*, 1975) reported EGF to cause distinct morphological alterations to cells.

FGFb was biologically more potent for the normal and sarcoid derived cells than FGFa. Fibroblast growth factors stimulate proliferation of most major cell types involved in wound healing both *in vivo* and *in vitro* (Cordon-cardo *et al.*, 1990). In addition, FGFb induces cell migration, neovascularisation and the formation of granulation tissue in animal models. In our studies we found both FGFa and FGFb to be inhibitory for the granulation tissue proliferation. However in the *in vivo* situation it could be that the granulation tissue fibroblasts within the tissue are producing excess FGFb which is in turn stimulating exuberant granulation tissue formation. This could be due to the fact that these cells have a larger number of receptors for FGFb or they may be influencing the secretion of other growth factors.

This study suggests that exuberant granulation tissue in the lower extremities of the horses limb might be regulated *in vivo* with the use of neutralising antibodies to TGFβ. This may enable the mechanisms of TGFβ release in the tissue to be down regulated by inhibiting the autocatalytic and autoinductive cascades of TGFβ amplification at the wound site, so reducing the eventual growth factor concentrations (Shah et al., 1992). The treatment of the exuberant granulation tissue *in vivo* with FGFb may be useful as a stimulant for the cells to release other growth factors. It may therefore be useful to measure quantities of TGFβ in the conditioned medium from both these cells and sarcoid-derived cells to establish if the levels are elevated from normal. Isolation and characterisation of these factors and others present in the conditioned medium may reveal

the presence of novel growth factors. Identification of these factors and the mechanisms that regulate their expression and activity will be important in understanding the processes of equine wound healing. In the next chapter the role of growth factors in the stimulation of the extracellular matrix by dermal fibroblasts *in vitro* is investigated.

CHAPTER SEVEN

The synthesis of extracellular matrix molecules by granulation tissue and sarcoid-derived dermal fibroblasts compared to normal equine dermal fibroblasts

7.1 INTRODUCTION

The main function of dermal fibroblasts is the synthesis of structural proteins, (in particular collagen) and reorganisation of the provisional extracellular matrix resulting in the formation of mature scar tissue.

In equine hyper-granulating wounds, the healing process is interrupted due to the overproduction of exuberant granulation tissue. Biochemical analysis (Chapter Four) indicated that there was an overproduction of collagen, especially type 1 in the exuberant granulation tissue.

Growth factors play an important role in the synthesis of newly formed extra cellular matrix molecules. TGFβ is a well known, potent stimulator of the production of a wide range of extracellular molecules (Rizzino, 1988). The effects of FGFs on collagen synthesis have been shown to depend on the cell type. It has been demonstrated that bFGF increased the proportion of collagen synthesised by endothelial cells (Tseng *et al.*, 1982).

The ability of dermal fibroblasts (derived from normal skin, granulation tissue and sarcoid lesions) to synthesise matrix molecules (collagen and protein) have been

investigated in culture. These investigations may give a indication of the metabolic reasons for the overproduction of granulation tissue in hyper- granulating wounds.

7.2 Methods

7.2.1 Source explantation and culture of dermal fibroblasts

Dermal fibroblasts were cultured from tissue described in Chapter 2, Table 2.2. The establishment of primary cell cultures and cell lines was carried out as described in Chapter 2, section 2.3.1. Media samples were collected from early passage cells, aliquoted and stored at -20°C for PICP and TGFβ analysis.

7.2.2 Collection of plasma and wound fluids

Normal peripheral blood was collected and processed from the horses mentioned in Chapter 2, section 2.5.2.

Wound fluid was collected from dressings that had been applied to non-healing wounds. The wound fluids were centrifuged and stored as described in Chapter 2, section 2.5.2.

7.6.3 Immunocytochemical analysis of extracellular matrix deposition, cell adhesion molecules and growth factors

Dermal fibroblasts were plated at a density of 2 x 10^4 cells/well on eight well culture slides (Gibco) in standard medium as described in Chapter 2, section 2.5.3. The cell cultures were stained immunocytochemically with monoclonal antibodies against extracellular matrix molecules, growth factors and cell adhesion molecules (Chapter 2, section 2.1.4.).

7.2.4 PICP procollagen determination

The PICP assay (Orion Diagnostica, Finland) is based on the standard radioimmunoassay. The method was described in Chapter 2, section 2.2.7.

7.2.5 TGFβ analysis

The quantities of TGFβ in the conditioned medium was measured using an ELISA method (Genzyme), as described in Chapter 2, section 2.2.8.

7.2.6 Lowry protein and hydroxyproline estimations

The quantities of protein and hydroxyproline were measured in serum and wound fluids from the horses described above. The methods used are described in chapter 2, sections 2.2.4 and 2.2.6 respectively.

7.2.7 Metabolic labelling of cell cultures

The ability of normal dermal fibroblasts, granulation tissue and sarcoid derived fibroblasts to synthesise collagen and other proteins was investigated. The method for metabolic labelling is described in Chapter 2, section 2.5.7.

7.2.8 Preparation of media and cell layer fractions

The conditioned media was removed from the cultures and the cell layer was prepared for assay as described in Chapter 2, section 2.5.8.

7.2.9 Analysis of synthesised protein

Protein synthesis was determined by the incorporation of [5-^{3}H]-proline into TCA precipitable counts, described in Chapter 2, section 2.5.9.

7.2.10 Analysis of synthesised collagen

Collagen was assayed by a modification of the procedure described by Peterkovsky & Diegelmann (1971), described in Chapter 2, section 2.5.10.

7.3 Results

7.3.1 Immunocytochemical analysis of extracellular matrix molecules in dermal fibroblasts

Fibroblasts from normal skin, sarcoids and chronically inflamed granulating wounds could be cultured from initial biopsy explants relatively easily. These cells were analysed immunocytochemically for extracellular matrix molecules.

A) Laminin

Most of the cells showed a bright intercellular staining for laminin and diffuse extracellular staining (Plate 7.1). There was no detectable difference in the intensity of staining for laminin between the cell types.

B) Fibronectin

All the cells had apparently equally intense staining for fibronectin. There was intracellular localisation of fibronectin in the perinuclear cytoplasm of the cells and strong intracellular staining around the nucleus in these cells (Plate 7.2).

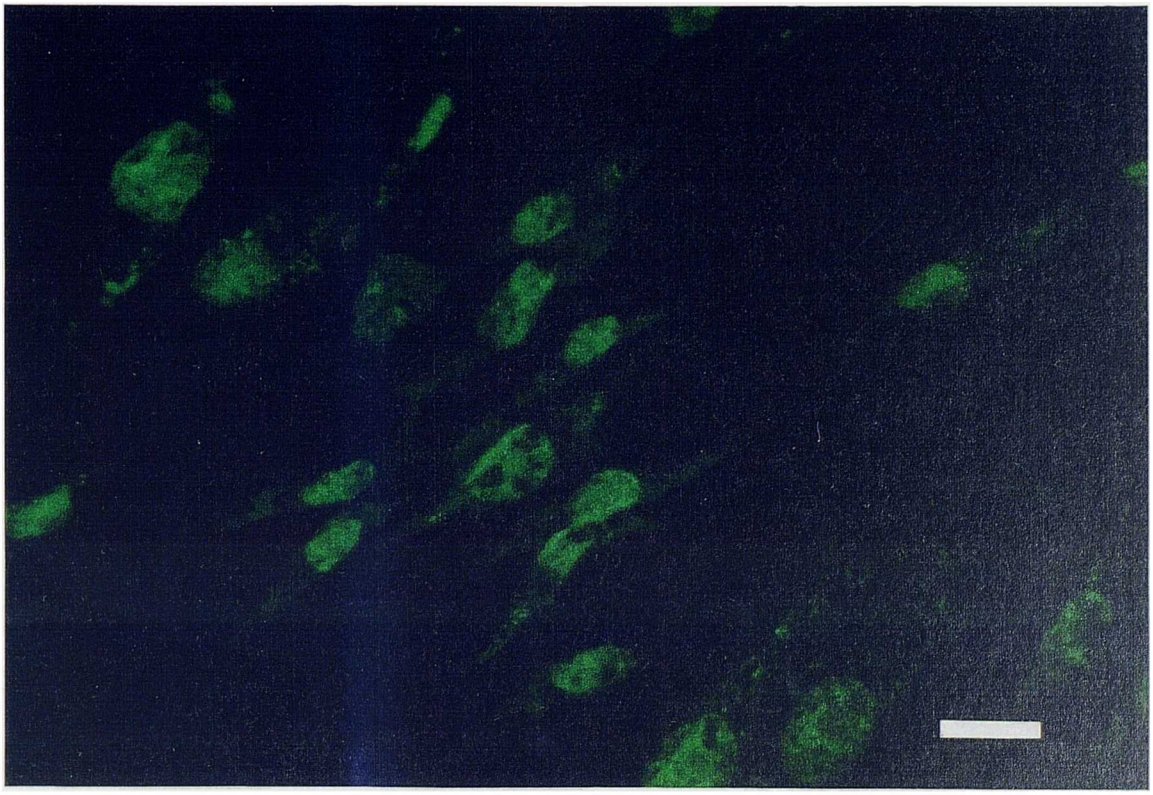


Plate 7.1 Photomicrograph of dermal fibroblasts stained for laminin Bar:100μm

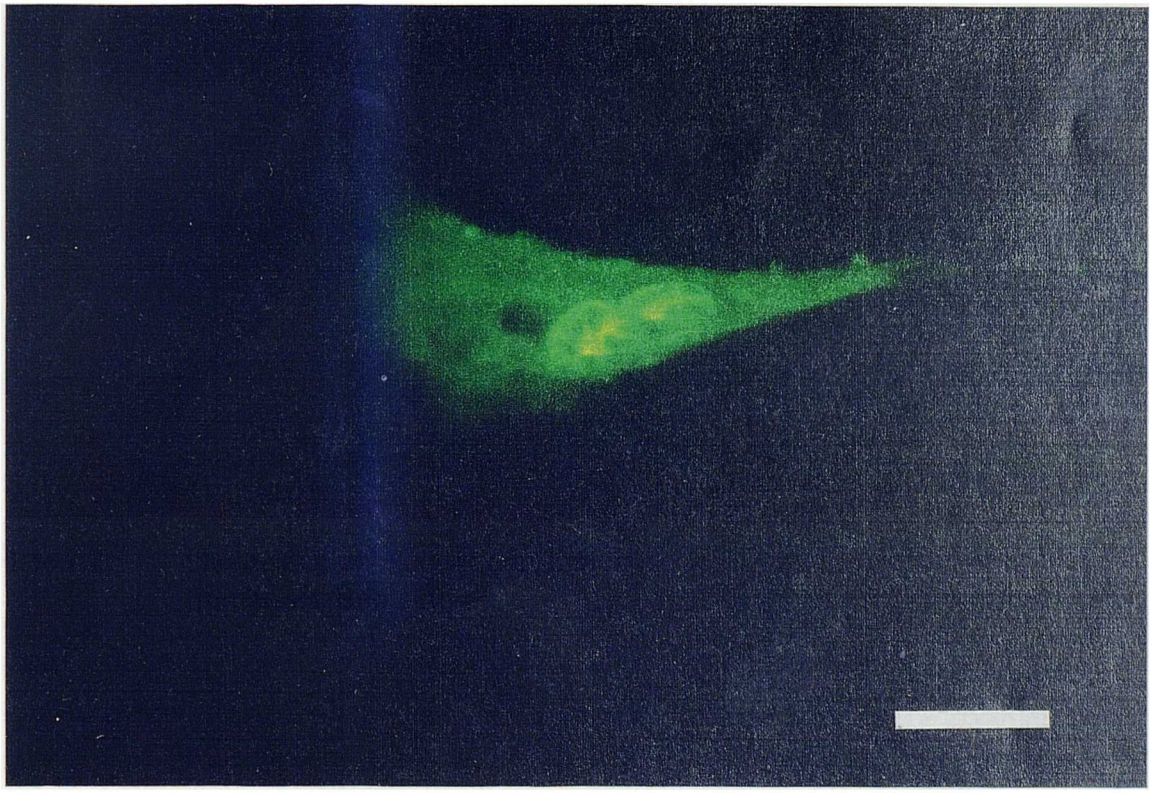


Plate 7.2 Photmicrograph showing the immunolocalisation of fibronectin in dermal fibroblasts Bar: 100μm

C) Collagen

Cells stained intracellularly for type I collagen with intense staining in the perinuclear and peripheral cytoplasm (Plate 7.3). Some cells showed intense fibrillar staining throughout the length of the cell.

Staining with type IV collagen showed a marked contrast to that of type I. A small percentage of cells stained intracellularly, staining was weak and located mainly around the nucleus (Plate 7.4). Extracellular staining was very diffuse with little evidence of the fibrous appearance noticed with type I collagen.

7.3.2 Immunocytochemical analysis of cell adhesion molecules in dermal fibroblasts

A) E-selectin

All the dermal fibroblasts stained intracellularly for E-selectin. Staining was very intense in perinuclear cytoplasm with very weak staining in the peripheral cytoplasm and extracellularly (Plate 7.5).

B) ICAM

A similar staining pattern to E-selectin was observed with ICAM. Although the perinuclear staining was much less intense with ICAM than it had been with E-selectin (Plate 7.6).

C) VCAM and PCAM

Faint staining was observed in the dermal fibroblasts with VCAM and PCAM. Intracellular staining was perinuclear and very weak. Extracellular staining was diffuse and weak in nature (Plate 7.7 and 7.8).

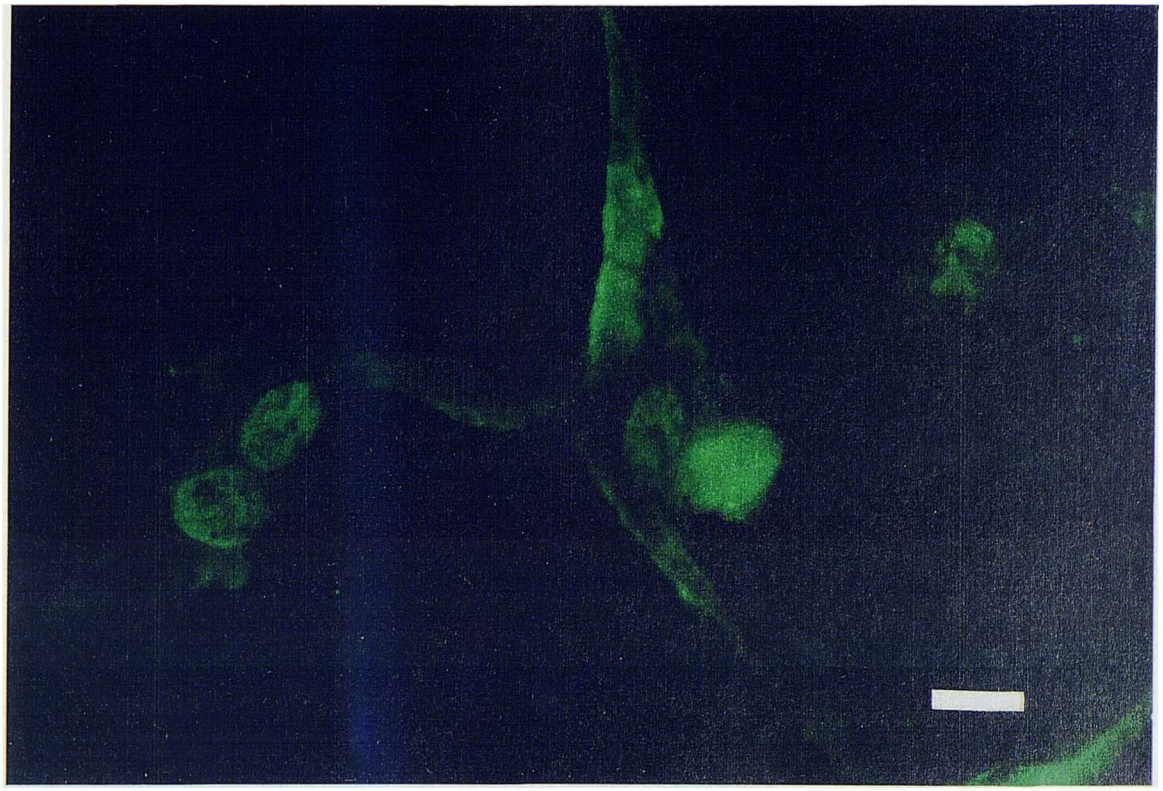


Plate 7.3 Photomicrograph showing immunolocalisation of type I collagen in dermal fibroblasts Bar: 100μm

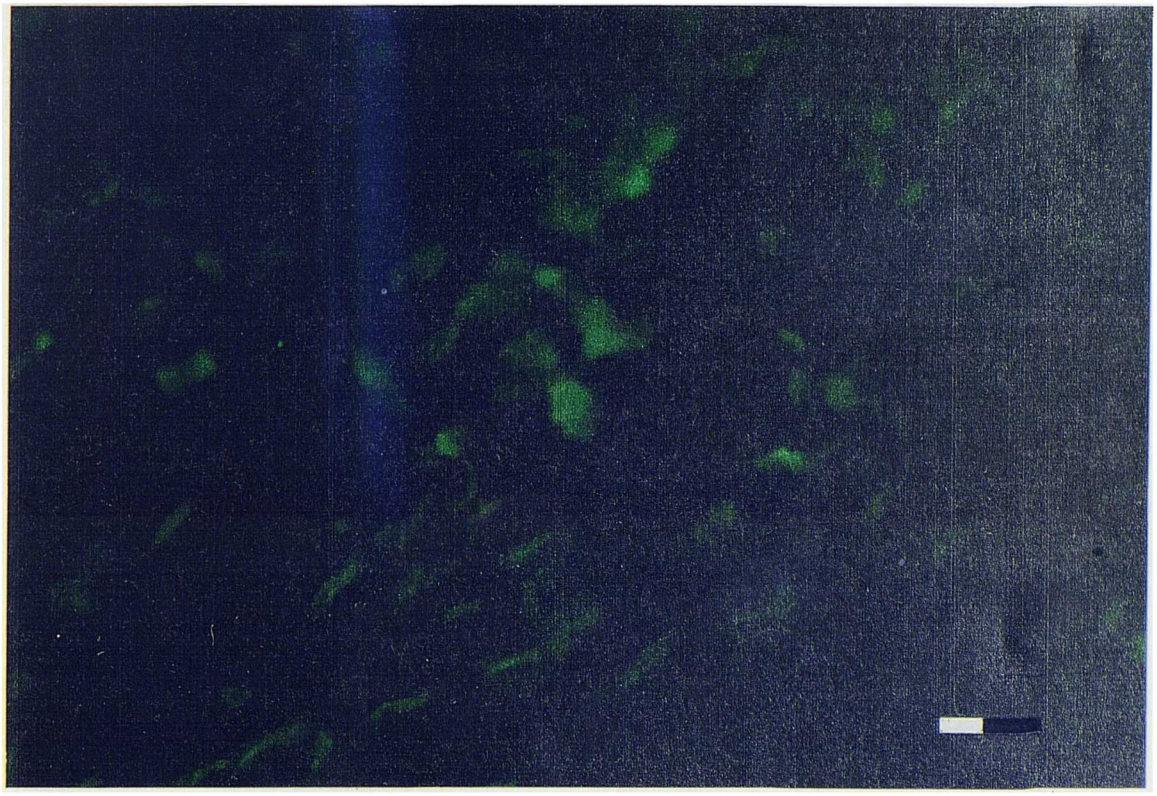


Plate 7.4 Photomicrograph showing the immunolocalisation of type IV collagen in dermal fibroblasts Bar: 100μm

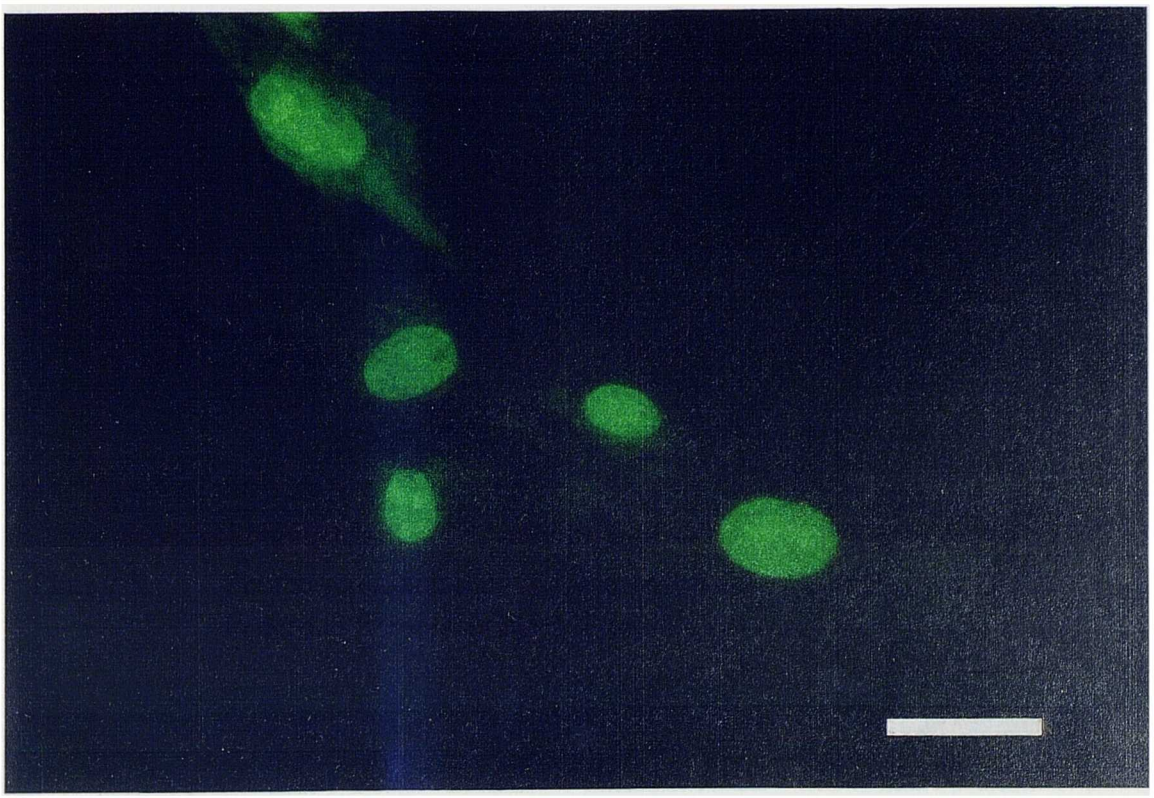


Plate 7.5 Photomicrograph showing immunolocalisation of E-Selectin in dermal fibroblasts Bar:100μm

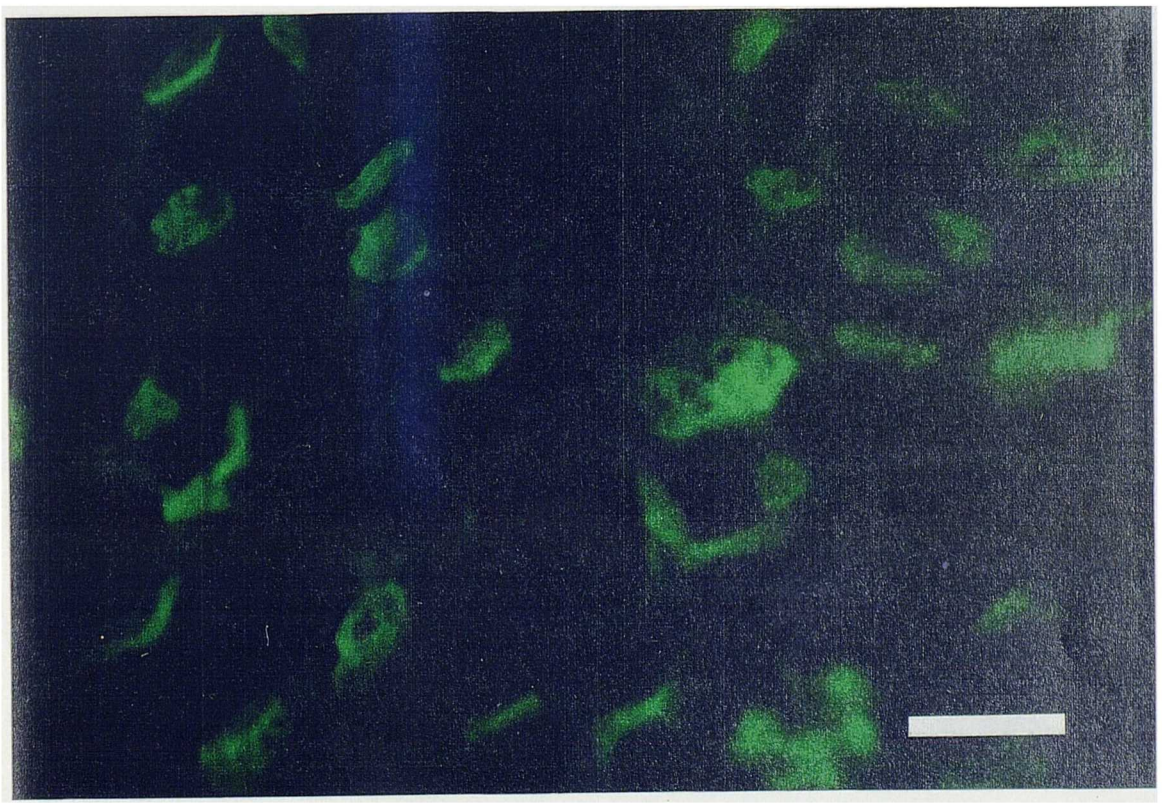


Plate 7.6 Photomicrograph showing immunolocalisation of ICAM-1 in dermal fibroblasts Bar: 100μm

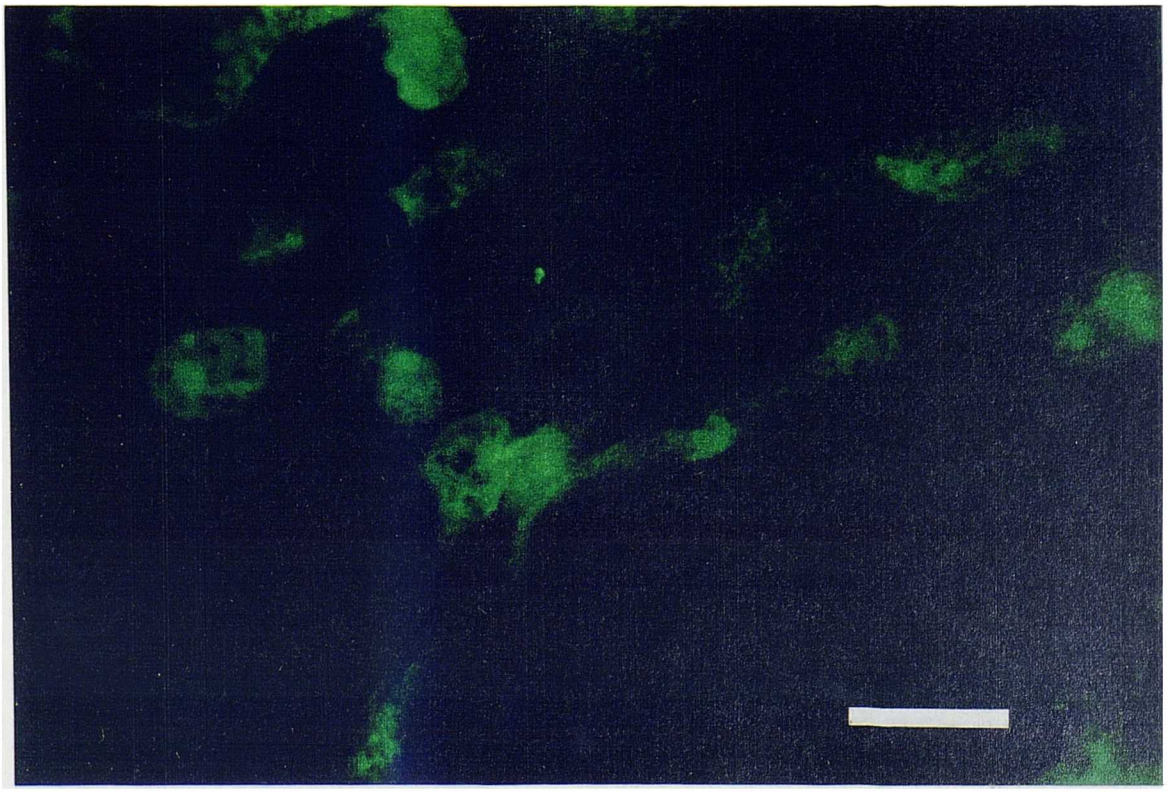


Plate 7.7 Photomicrograph showing immunolocalisation of VCAM in dermal fibroblasts Bar:100μm

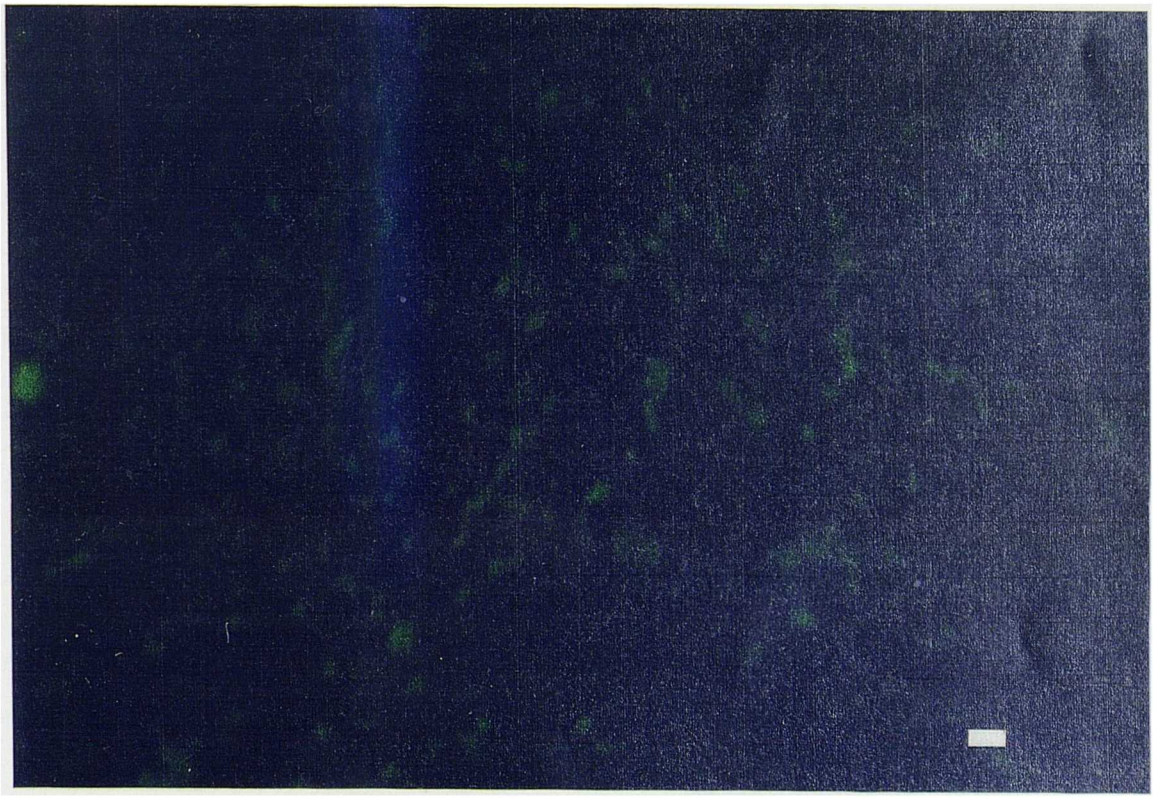


Plate 7.8 Photomicrograph showing the immuolocalisation of PCAM in dermal fibroblasts Bar: 100μm

7.3.3 Immunocytochemical examination of growth factor localisation in dermal fibroblasts

A) Epidermal growth factor (EGF)

EGF was localised in the perinuclear cytoplasm of the cells, where staining was relatively weak. The peripheral cytoplasm stained extremely weakly and there was no extracellular staining for EGF at all (Plate 7.9).

B) Fibroblast growth factor (FGF)

Strong intracellular staining was identified throughout the entire cell cytoplasm in all three cell types (Plate 7.10).

C) Platelet-derived growth factor (PDGF)

Staining with PDGF was very faint in the perinuclear and peripheral cytoplasm of the cells (Plate 7.11). There was also very faint fibrillar extracellular staining present.

D) Transforming growth factor beta (TGFβ)

Faint intracellular staining was seen when the cells were stained with antibodies to TGFβ. The staining in the perinuclear cytoplasm appeared to be 'grainy' and the peripheral cytoplasm stained very weakly (Plate 7.12).

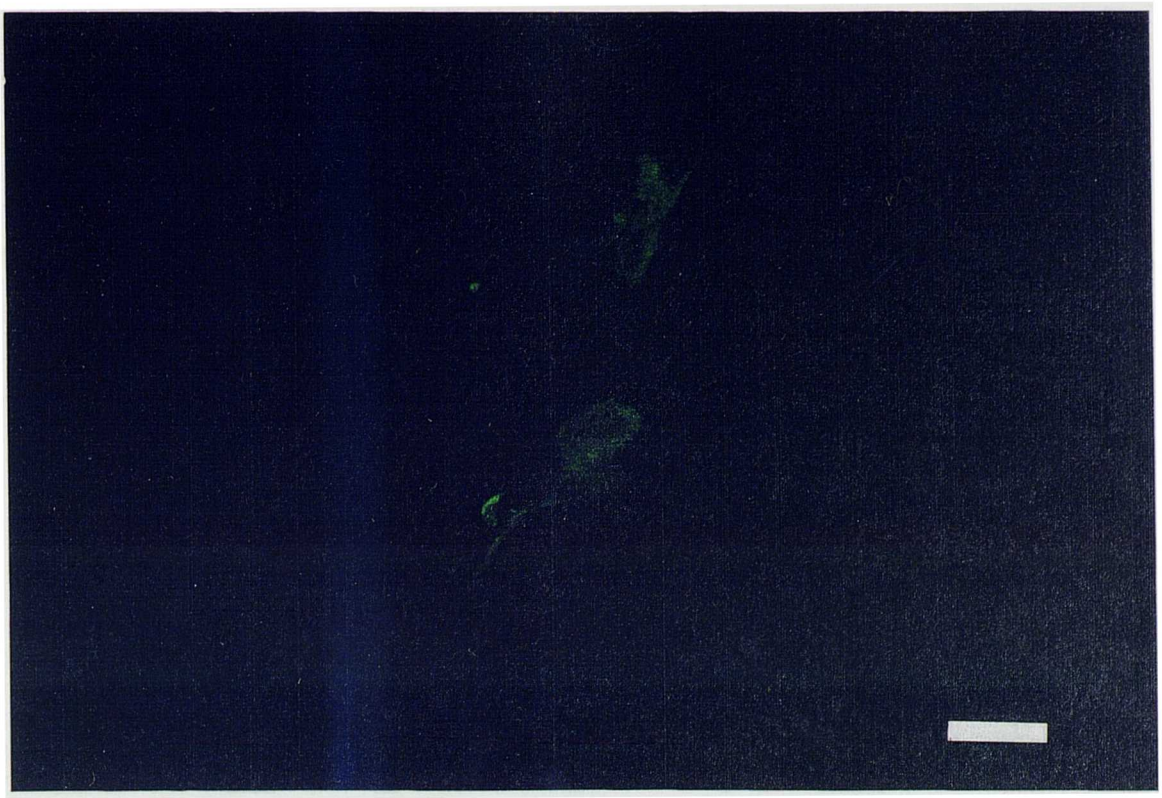


Plate 7.9 Photomicrograph showing the immunolocalisation of EGF in dermal fibroblasts Bar: 100μm

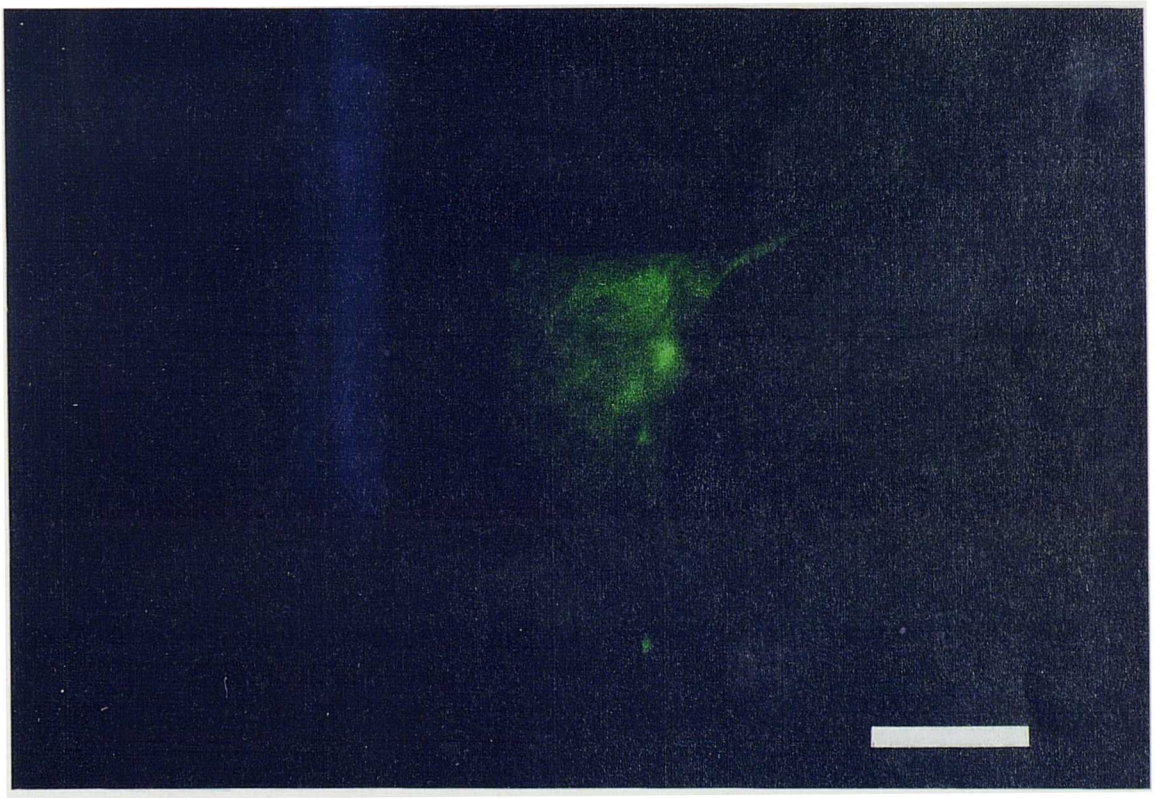


Plate 7.10 Photomicrograph showing the immunolocalisation of FGF in dermal fibroblasts Bar: 100μm

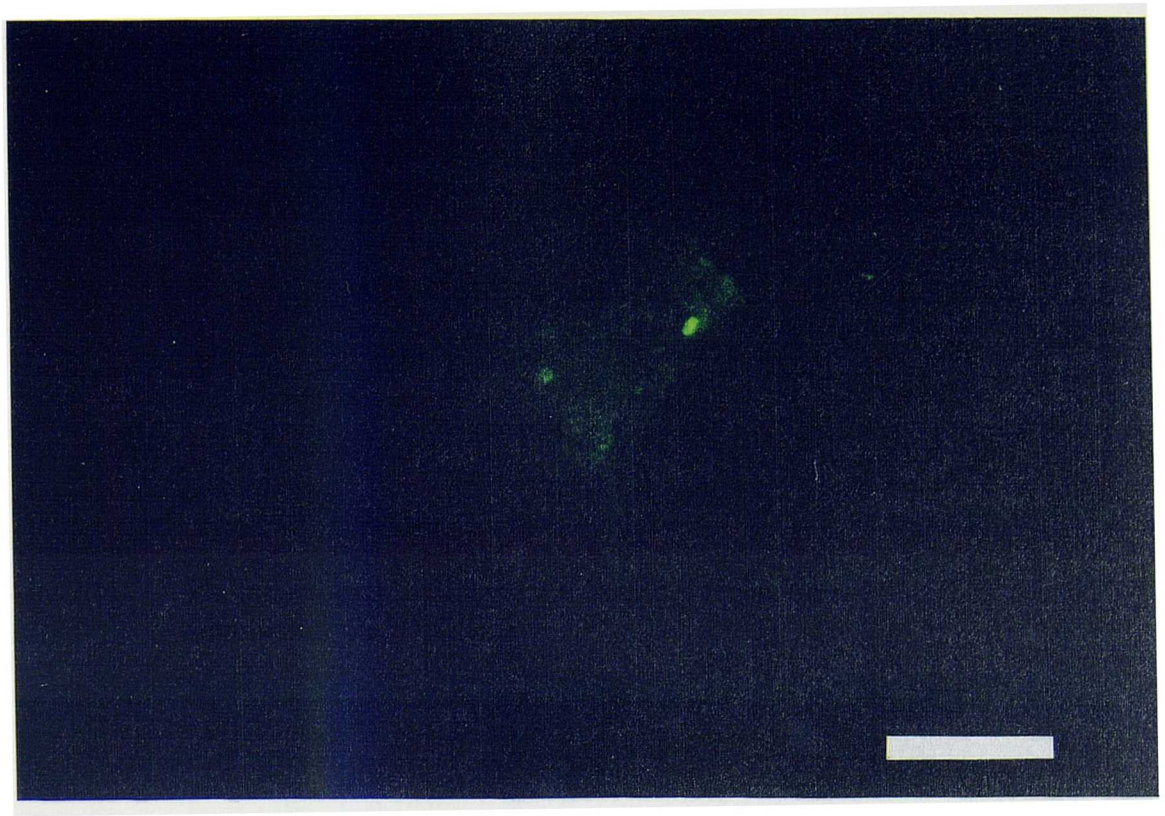


Plate 7.11 Photomicrograph showing the immunolocalisation of PDGF in dermal fibroblasts Bar: 100μm

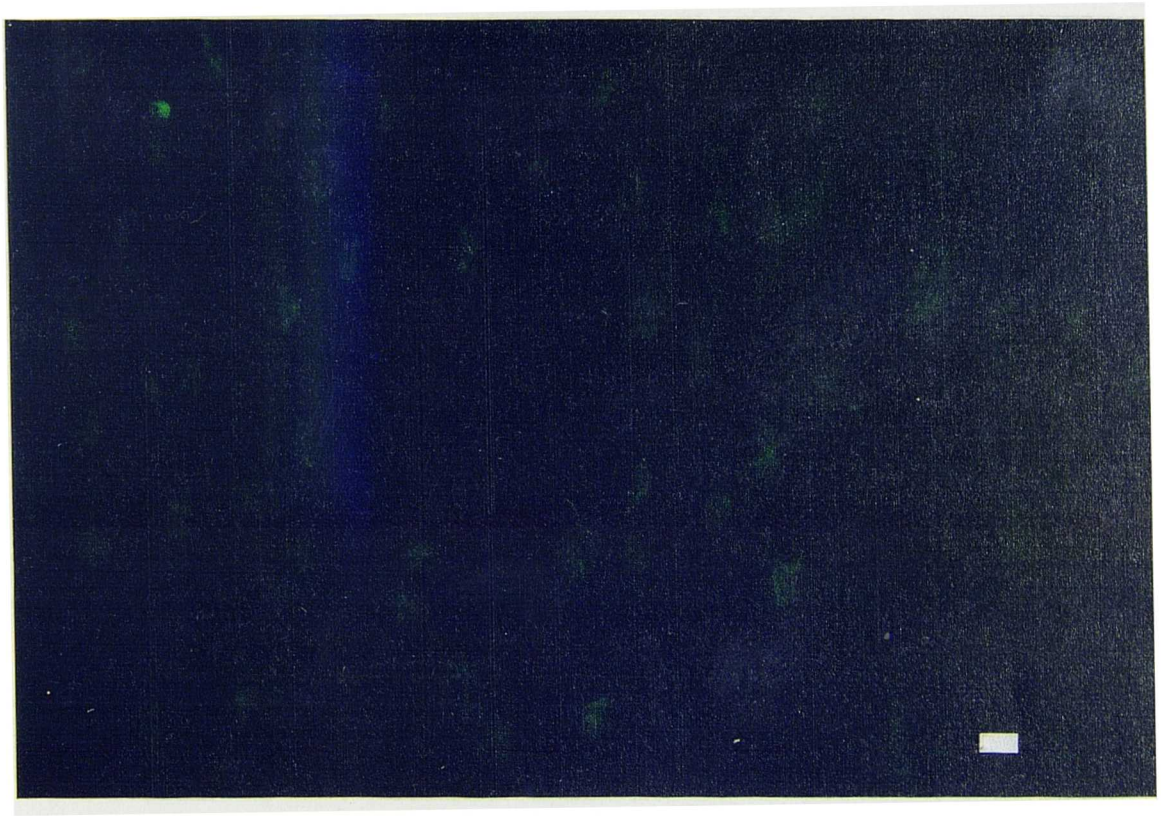


Plate 7.12 Photomicrograph showing the immunolocalisation of TGFβ in dermal fibroblasts Bar: 100μm

7.3.4 Type I procollagen (PICP) estimation

The concentrations of PICP type I procollagen was measured in plasma from healthy horses, sarcoid cases and horses with non-healing wounds (Figure 7.1). The values of PICP (mg/L) were not significantly different from each other.

Figure 7.2 illustrates the difference of the PICP concentration found in wound fluid, which was significantly higher than the control ($p<0.05$).

In the cell supernatants (Figure 7.3), there was a significant increase in PICP concentrations in the supernatant from granulation tissue cells ($P<0.05$) compared with that from the normal cells. A smaller increase was seen between the sarcoid-derived cell supernatants and the normal cell supernatants ($p<0.02$).

7.3.5 TGFβ determination

The levels of TGFβ1 found in the plasma of normal, sarcoid and wounded horses, was measured as previously described. Figure 7.4 shows that statistically there was no significant difference between the three groups.

The concentration of TGFβ1 was measured in wound fluids from horses with chronic non-healing wounds (Figure 7.5). The levels of TGFβ1 were significantly higher in the wound fluid samples when compared to the control ($p<0.05$).

Cells from normal skin, sarcoid tumours and granulating non-healing wounds were cultured as previously described. The supernatants were collected and the TGFβ1 levels were measured as described above. The values for the granulation tissue-derived cells were significantly higher ($p<0.005$) than the normal cells (Figure 7.6). However there was no significant difference between the normal and the sarcoid-derived cells.

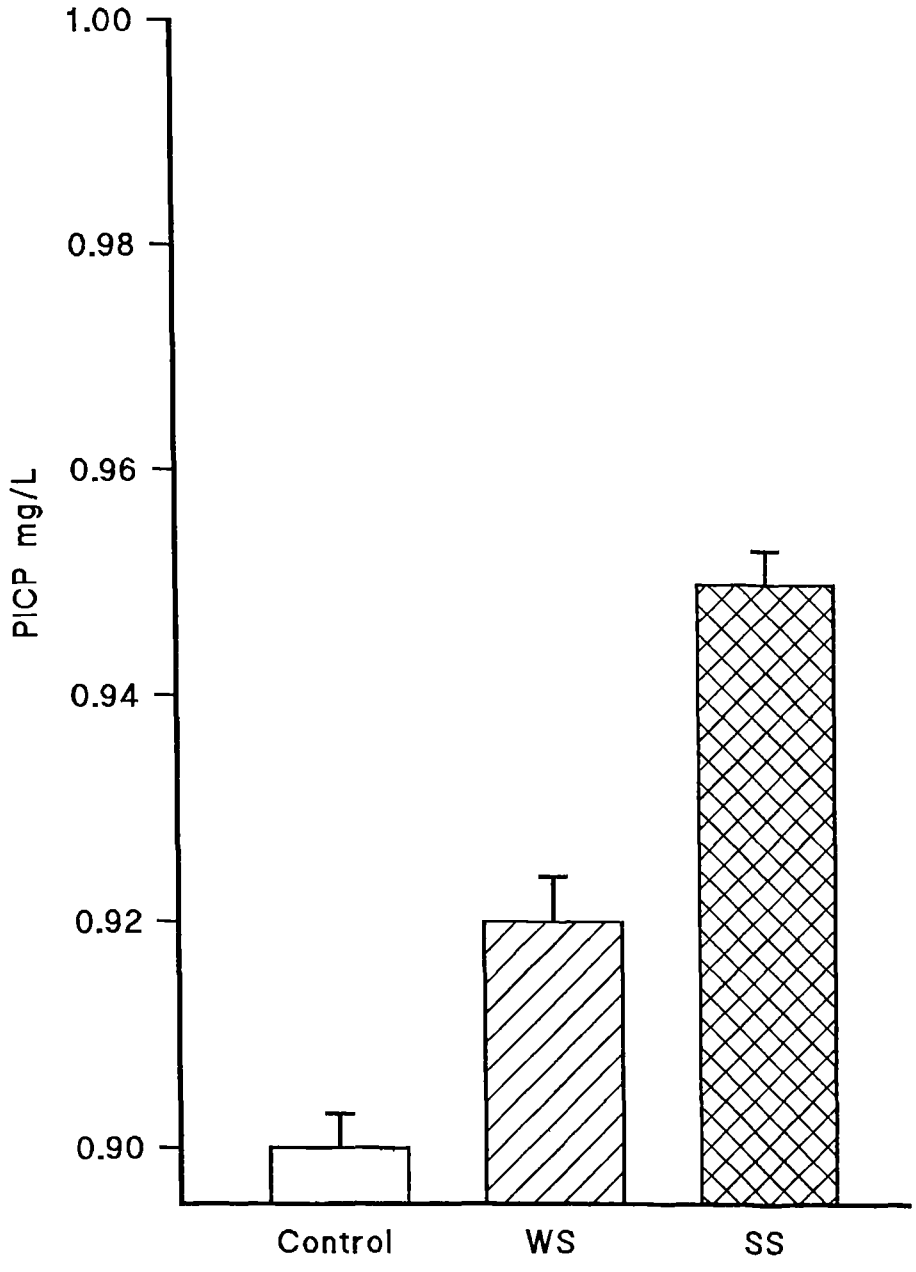


Figure 7.1 Concentration of type I procollagen (PICP) in plasma from control horse (□), horse with non-healing wound (▨) and horse with sarcoids (▩); (n=3).

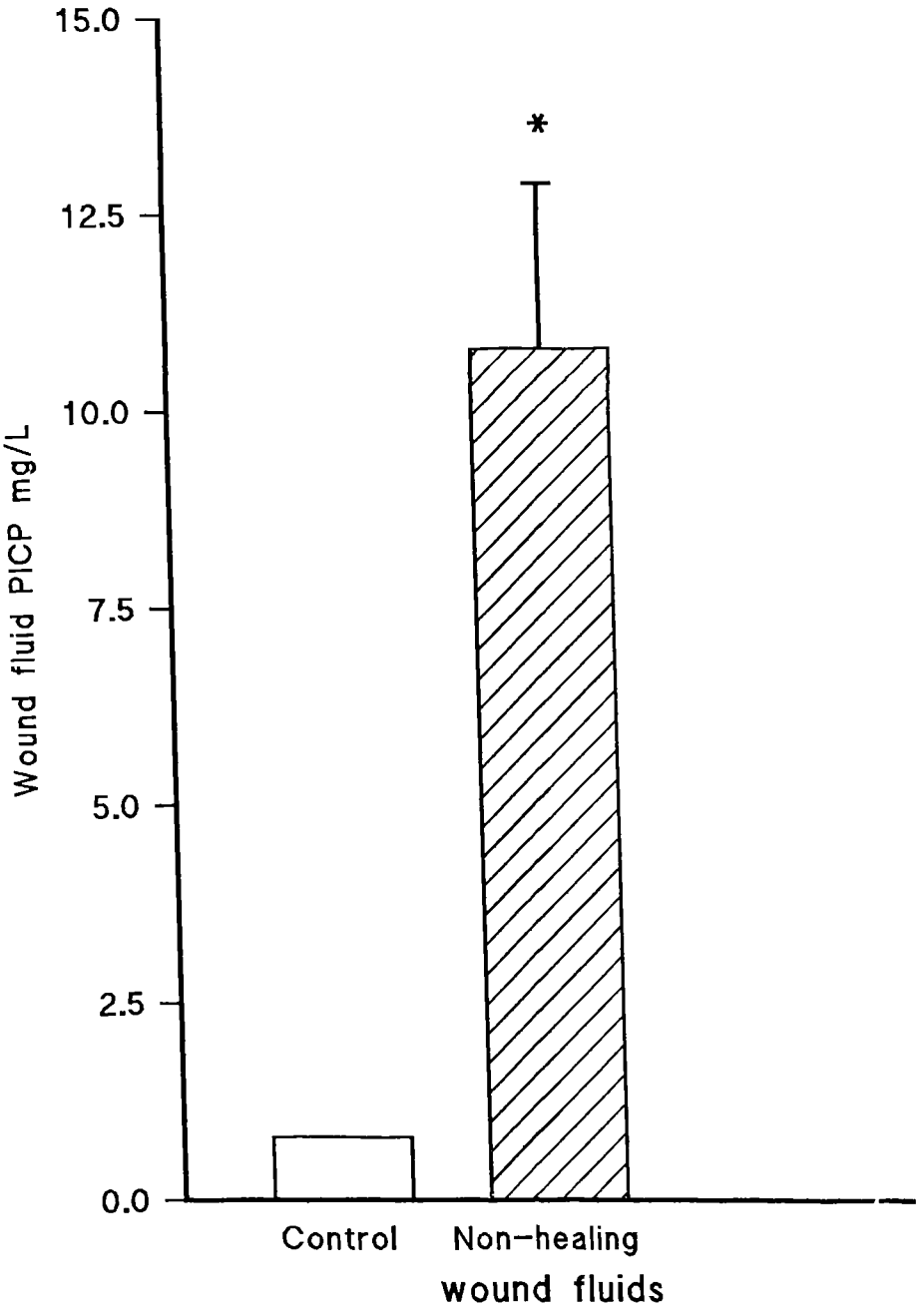


Figure 7.2 Concentration of type I procollagen (PICP) in wound fluids from control horse (□), horse with non-healing wound (▨) n=3. Values significantly different to control and non-healing wound * $P<0.05$.

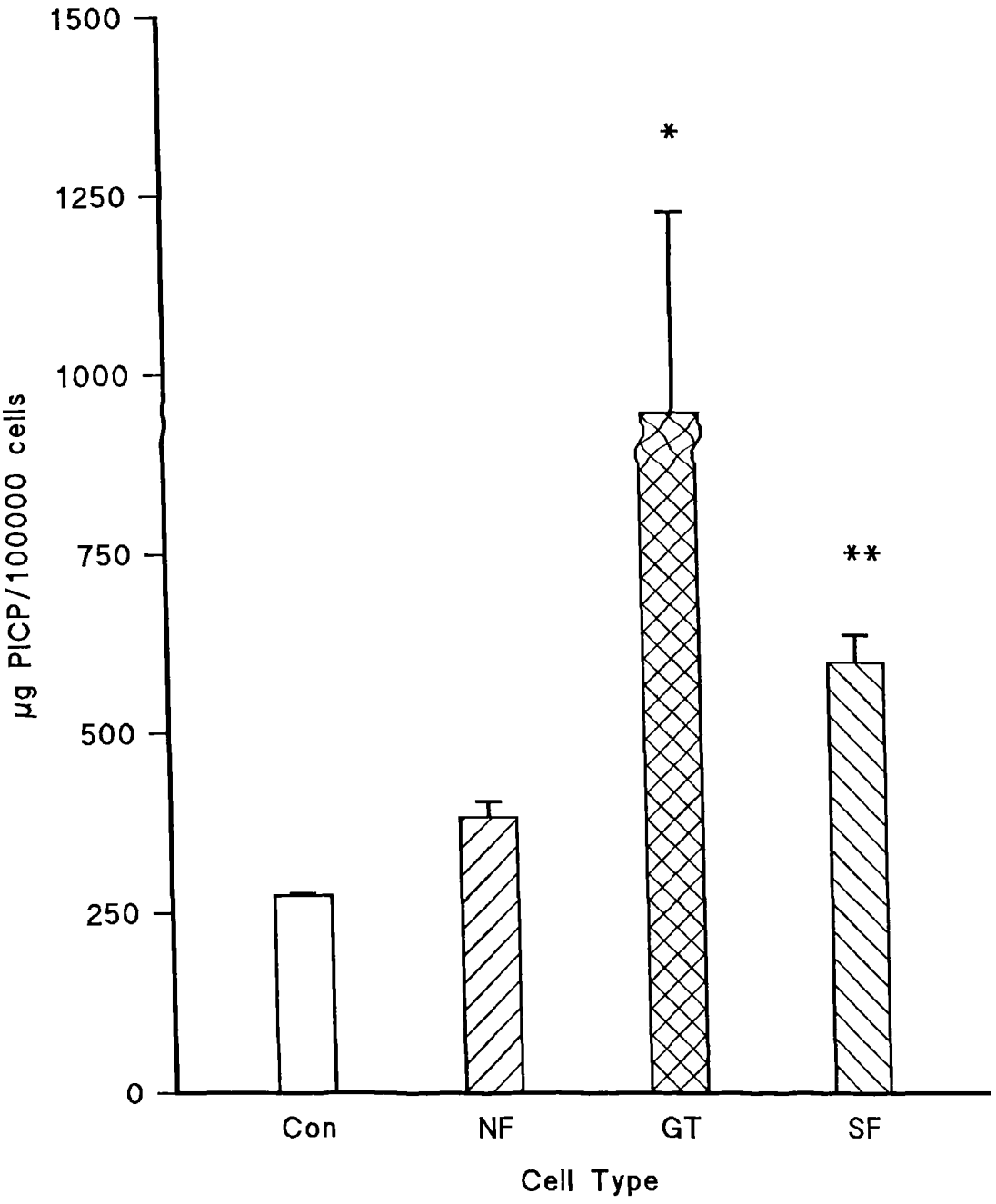


Figure 7.3 Concentration of type I procollagen (PICP) in cell supernatants. Control, media containing 2.5% FCS (□); n=4; granulation tissue cells (▨); n=5; normal fibroblasts (▨); n=6; sarcoid cells (▨); n=4. Values significantly different between NF and GT * $P<0.05$; NF and SF, ** $P<0.02$.

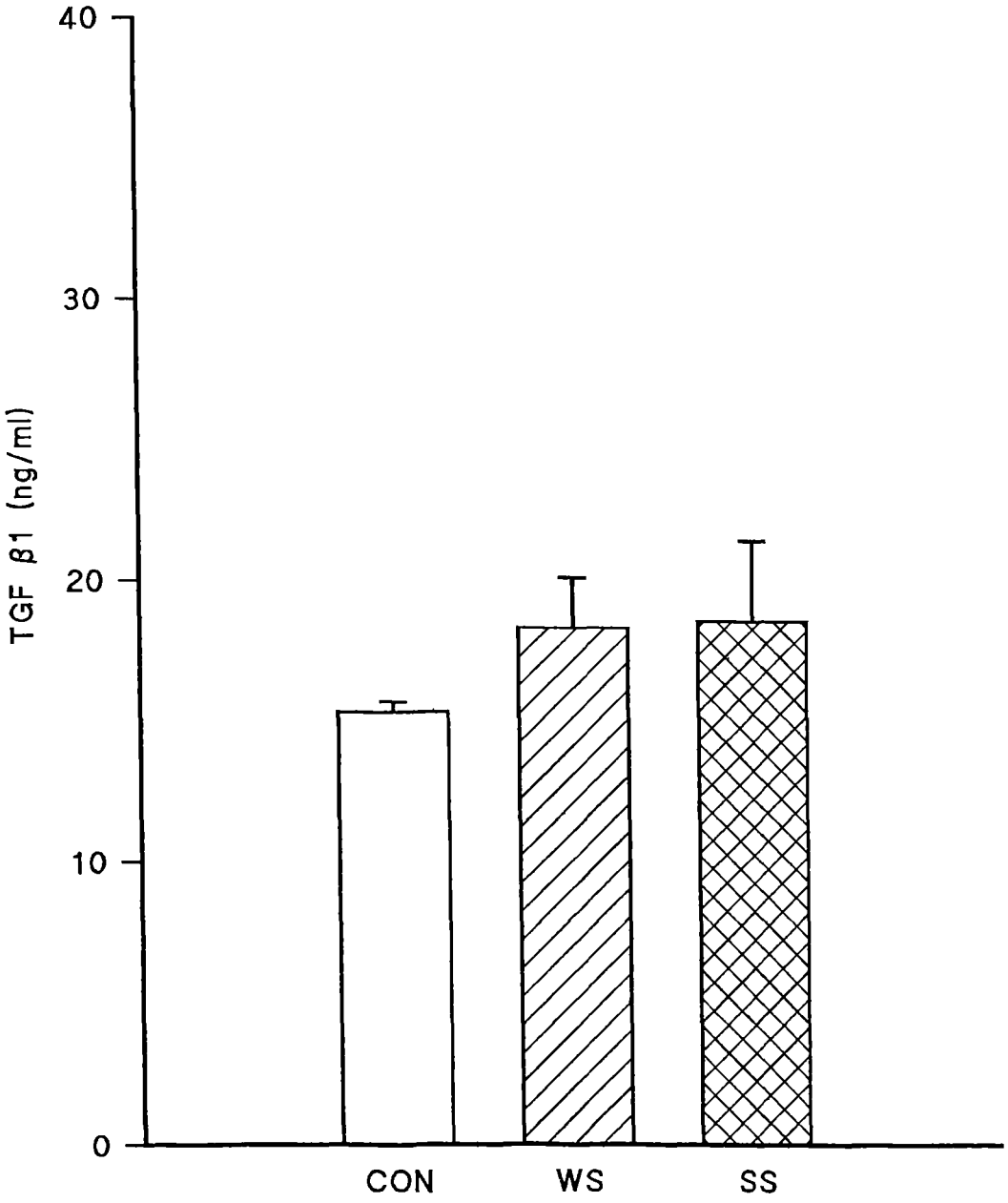


Figure 7.4 Concentration of TGFβ1 in PLASMA from control horses (□); n=3; PLASMA from horses with non-healing wounds (▨); n=5; PLASMA from horses with sarcoids (▩); n=3.

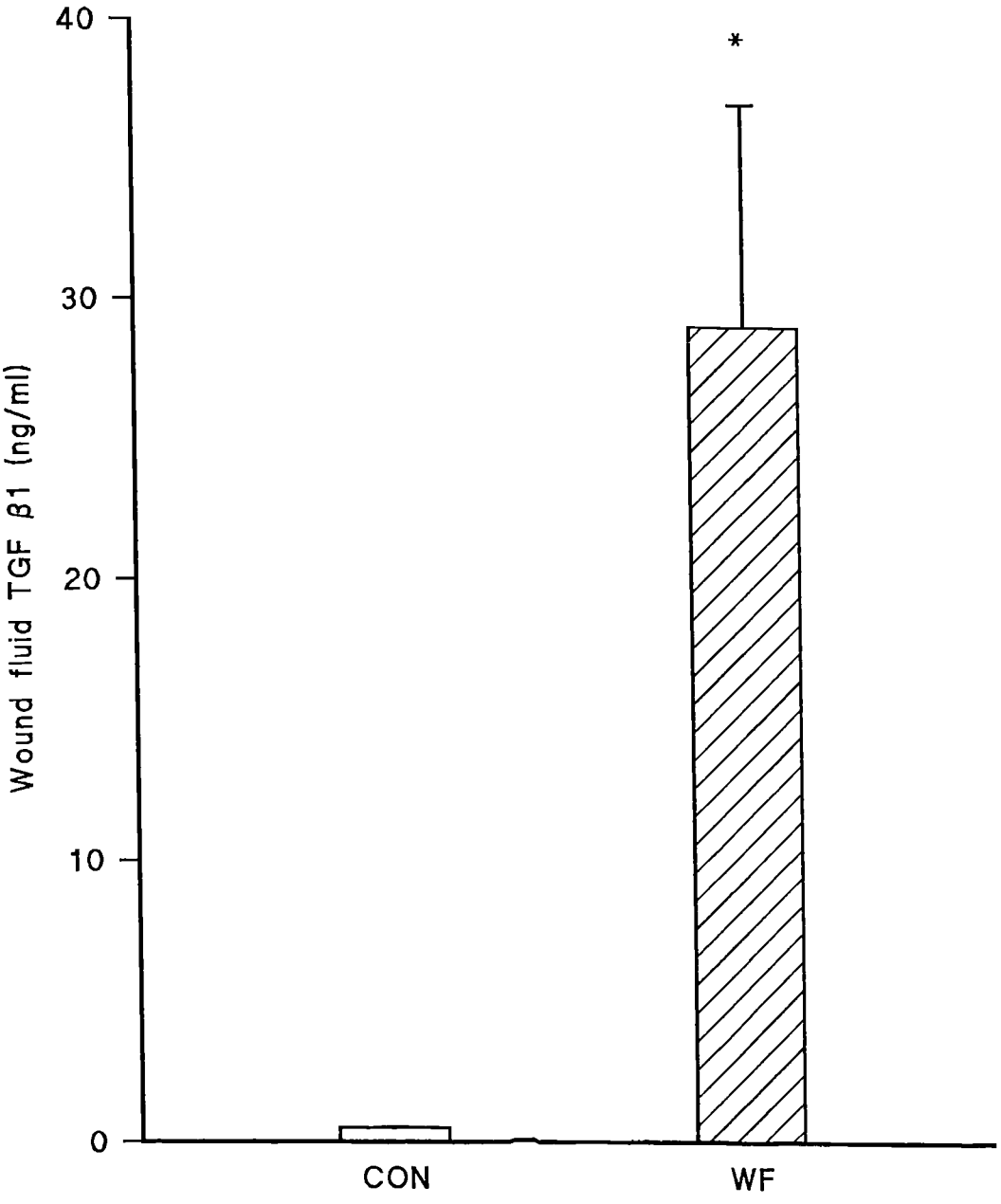


Figure 7.5 Concentration of TGFβ1 in wound fluids. Control horses (□); n=3; horses with non-healing wounds (▨); n=4. Values significantly different between control and wound fluid * $P<0.05$.

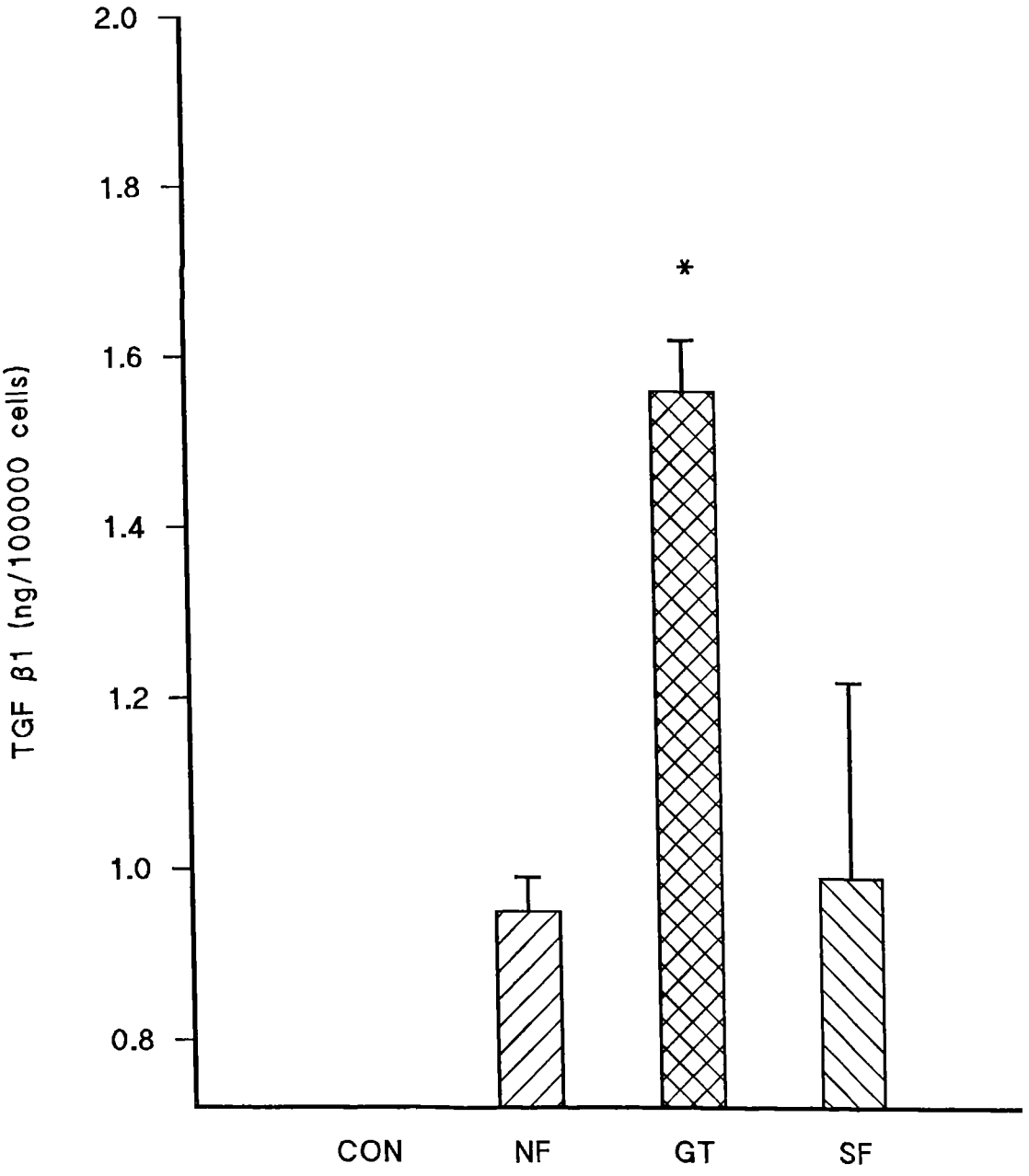


Figure 7.6 Concentration of TGFβ1 in cell supernatants containing 2.5% FCS. Control (□); n=3; normal cells (▨); n=6; granulation tissue-derived cells (▩)N=5; sarcoid cells (▧); n=5. Values significantly different between NF and GT * P<0.005.

7.3.6 Protein content of plasma and wound fluid

The levels of protein in plasma and wound fluid from normal, sarcoid and chronic non-healing wounded horses was measured using the Lowry method as described in Chapter 2. The protein concentration in the plasma from the three groups was very similar, however, the levels in the plasma from the granulating wounds tended to be higher than the sarcoid and normal plasma although this was not significant (Figure 7.7).

The concentration of protein found in wound fluid samples was compared with a control (Figure 7.8). The wound fluids were found to be significantly higher than the control ($p<0.002$).

7.3.7 Hydroxyproline estimation

Hydroxyproline was measured in serum and wound fluids from horses previously described using the method reported in Chapter 2. Figure 7.9 demonstrates that there were slightly lower levels in the sarcoid serum compared to the control normal serum ($p<0.02$). Likewise slightly lower levels of hydroxyproline were observed in the serum from wounded horses ($p<0.05$).

The concentration of hydroxyproline in the wound fluid was however, was not significantly higher than the control (Figure 7.10).

7.3.8 Effect of growth factors on protein biosynthesis

Incorporation of [^{3}H]-proline into collagenous and non-collagenous protein by normal, sarcoid and granulation tissue-derived fibroblasts was compared following treatment with EGF, FGFa&b and TGFβ. The six different sites in the horse described previously, were investigated.

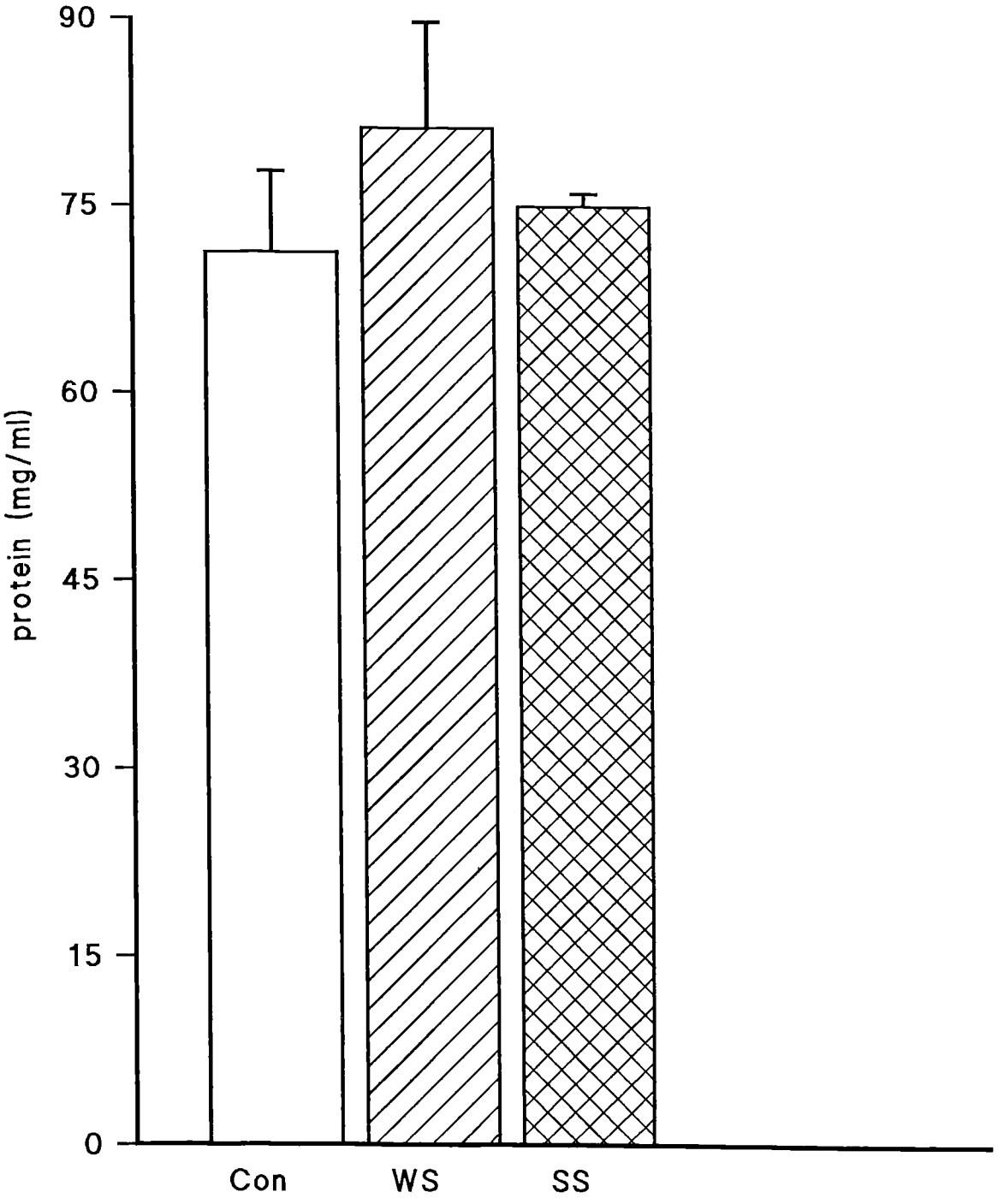


Figure 7.7 Concentration of protein from [illegible]. Control [illegible] (▭); n=3; [illegible] from wounded horses (▨); n=5; [illegible] from horses with sarcoids (▩); n=4.

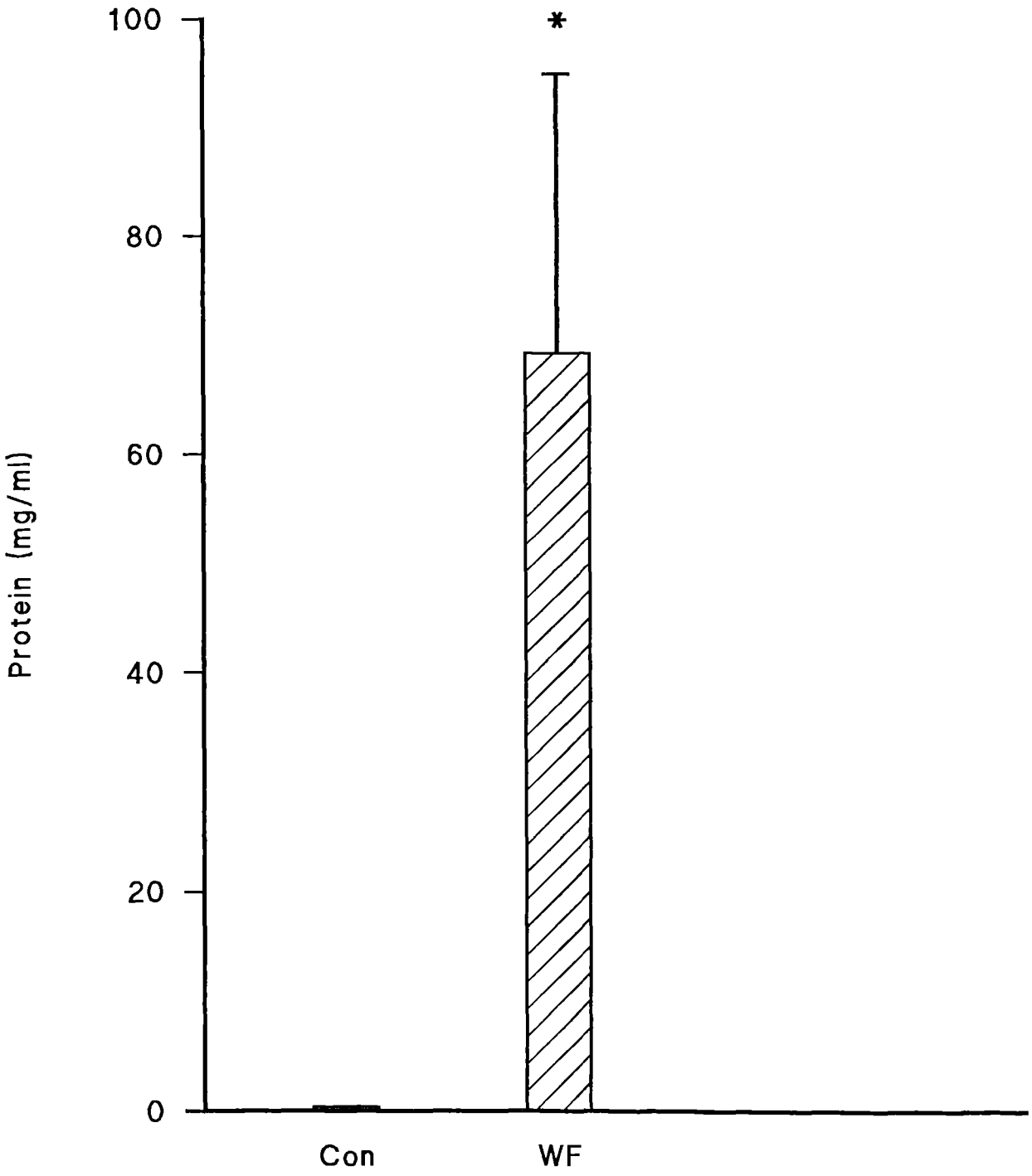


Figure 7.8 Concentration of protein in wound fluids. Control (□); n=3; wound fluid from horses with non-healing wounds (▨); n=5. Wound fluid values differed significantly from the control * P<0.002.

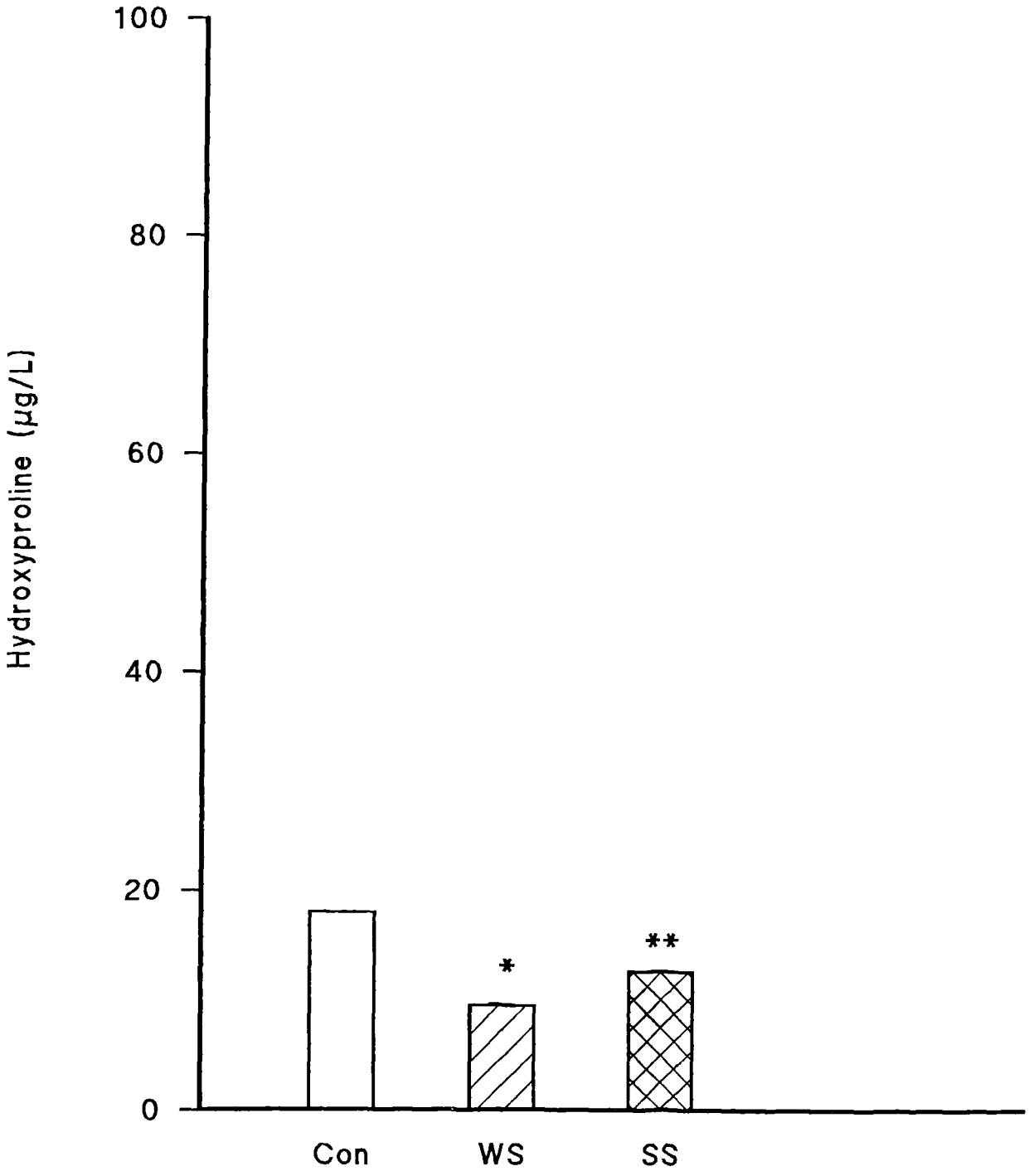


Figure 7.9 Concentration of hydroxyproline in [illegible] from control horses (□); n=3; plasma from wounded horses (▨); n=4; plasma from horses with sarcoids (▩); n=4. Values significantly different in control and wounded horses * P<0.05; control and sarcoid ** P<0.02.

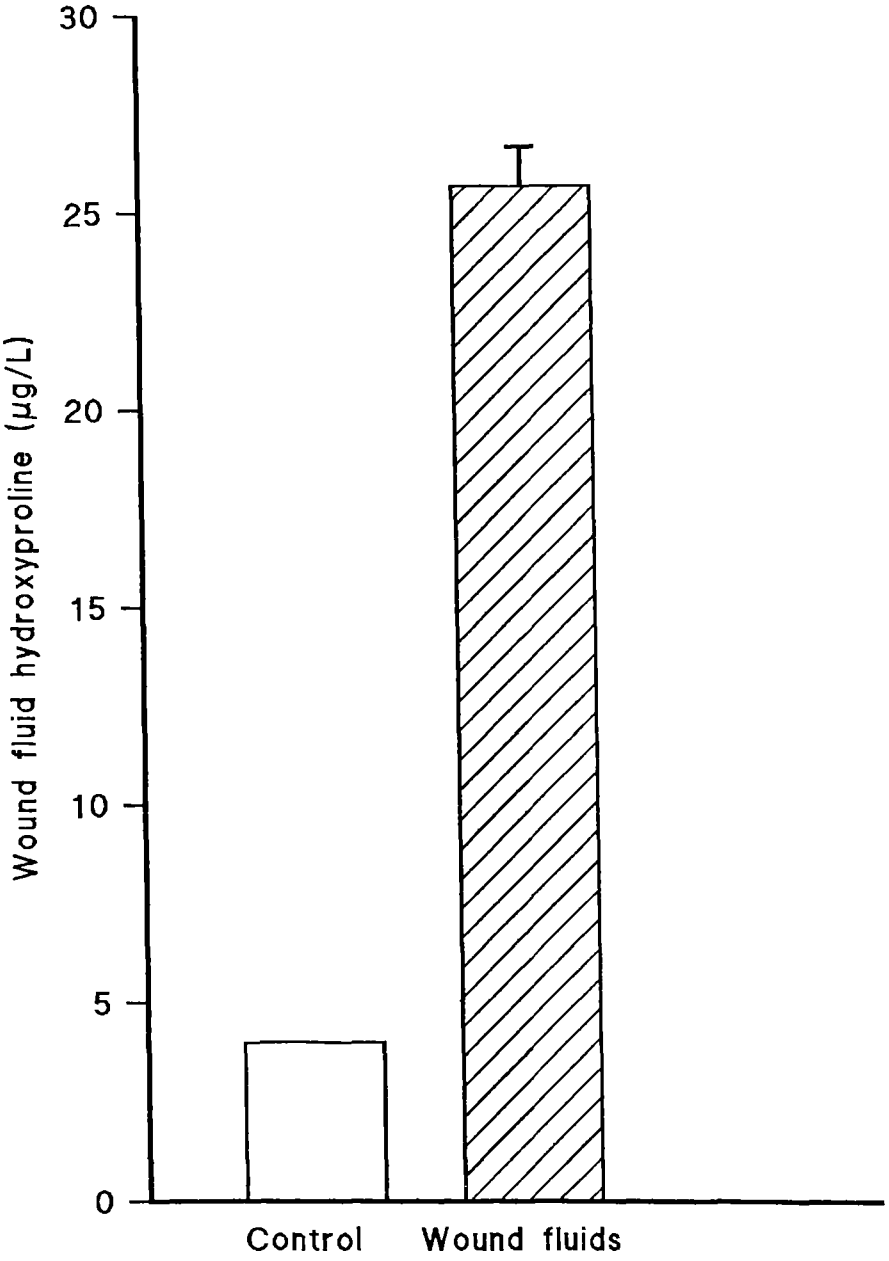


Figure 7.10 Concentration of hydroxyproline in wound fluids. Control horses (□); n=3; wound fluid from horses with non-healing wounds (▨); n=4.

A) Protein synthesis by dermal fibroblasts treated with EGF

Incorporation of [^{3}H]-proline into the total protein released into the cell supernatants of untreated control, normal, granulation tissue and sarcoid derived cells is shown in Figure 7.11. The total protein released into the medium of these cells with the addition of EGF (10ng/ml) is shown in Figure 7.11a. Protein released into the media of granulation tissue derived cells was significantly lower than the normal cells ($p<0.02$). Figure 7.12a demonstrates the incorporation of [^{3}H]-proline into the total protein of the cell extracts of the above cells treated with EGF. The protein in the granulation tissue cell extracts was significantly lower than the normal cells ($p<0.0001$).

The total protein was measured in the cell supernatants and extracts of cells taken from the six different sites of the horse that have been previously described. The data in Figure 7.13a shows that there was no significant difference between the cell supernatants from any of the six sites. Likewise, Figure 7.14a shows that there was no significant difference between the six sites in the cell extracts.

B) Protein synthesis of dermal fibroblasts treated with FGFa

The protein content of cell supernatants and extracts, treated with FGFa 10ng/ml) were measured as previously described. Figure 7.11b shows that the protein released into the medium of the granulation tissue-derived cells was significantly lower than the normal cells ($p<0.001$) and significantly lower than the sarcoid-derived cells ($p<0.02$). A similar pattern was seen in the cell extracts (Figure 7.12b) where the granulation tissue-derived cells were significantly lower than the normal cells ($p<0.01$) and the sarcoid cells ($p<0.05$).

The data in Figure 7.13b shows that there was no significant difference between

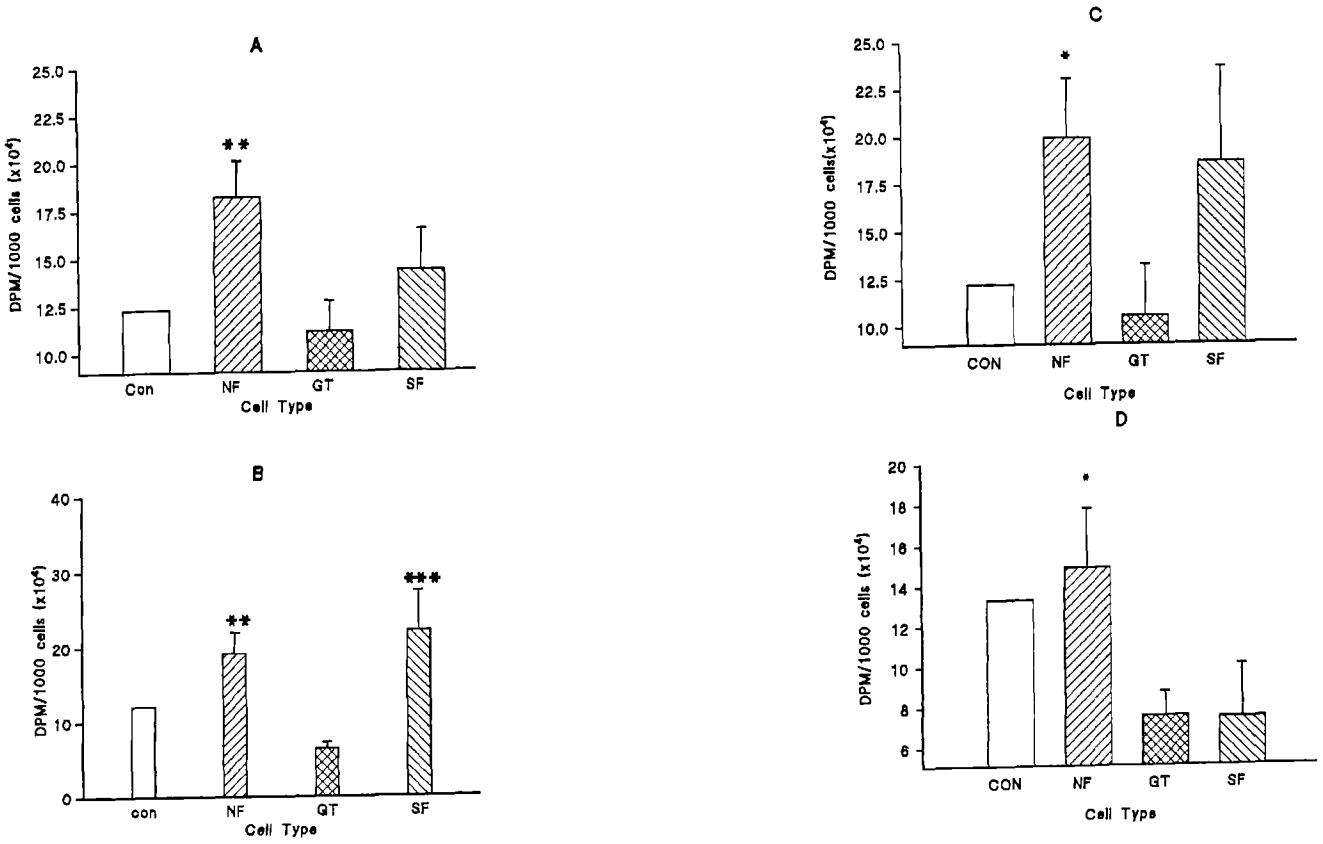


Figure 7.11 Incorporation of [3H] proline into the total protein of cell supernatants. Untreated control cells (□); n=3; normal treated fibroblasts (▨); n=4; granulation tissue treated cells (▩); n=4; and sarcoid treated cells (▧); n=4. A: treated with EGF (10ng/ml); B: FGFa (10ng/ml); C: FGFb (10ng/ml) and D: TGFβ (1ng/ml). Values significantly different to normal cells and granulation tissue cells * $P<0.05$; ** $P<0.001$ and sarcoid cells *** $P<0.02$.

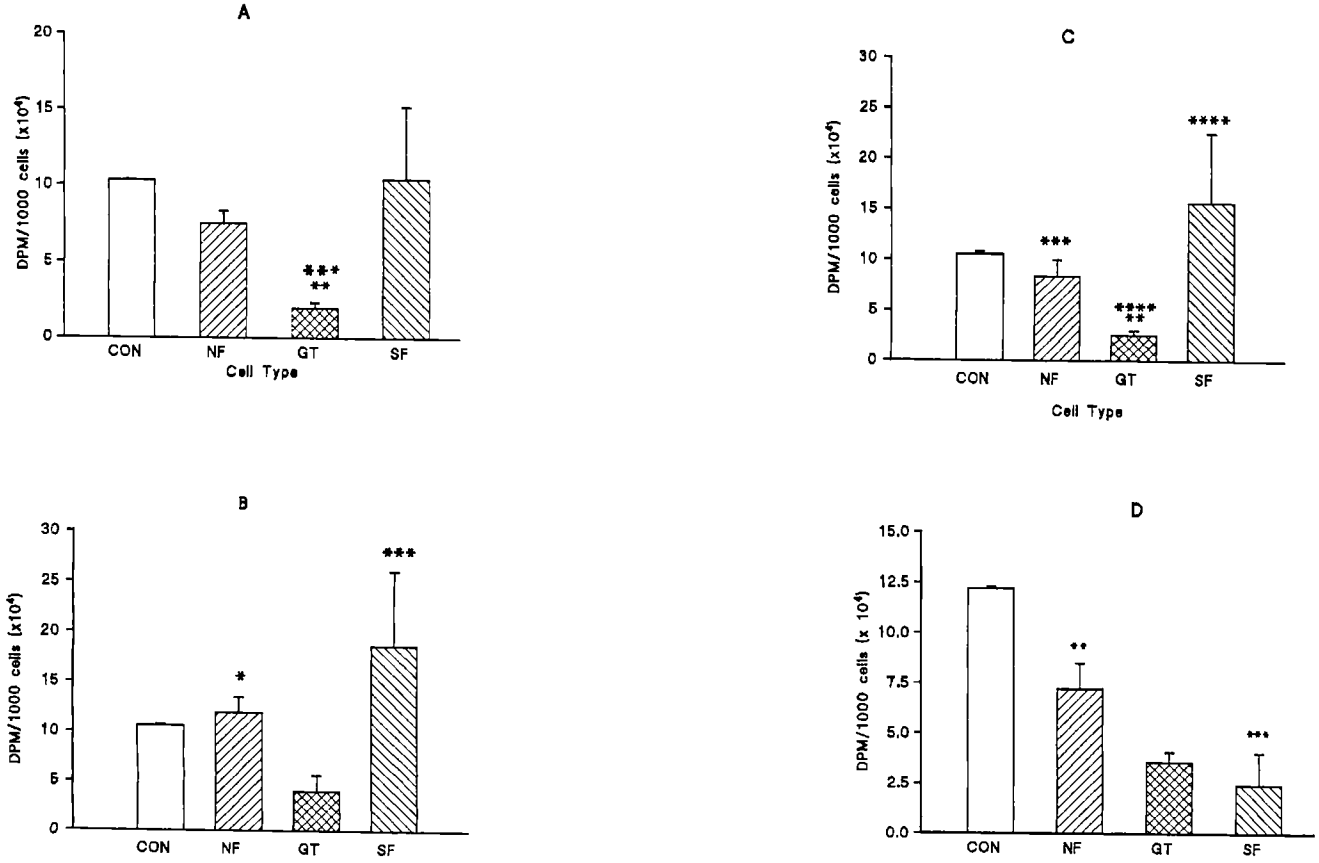


Figure 7.12 Incorporation of [3H] proline into the total protein of cell extracts. Untreated control cells (□); n=3; normal treated fibroblasts (▨); n=4; granulation tissue cells (▩); n=4; and sarcoid treated cells (▧); n=4. A: treated with EGF (10ng/ml); B: FGFa (10ng/ml); C: FGFb (10ng/ml) and D: TGFβ (1ng/ml). Values significantly different to normal cells and granulation tissue cells * P<0.01; ** P<0.02; ***** P<0.0001; ****** P<0.005 and sarcoid cells *** P<0.005; **** P<0.001.

the cell supernatants from any of the six sites. Likewise, Figure 7.14b shows that there was no significant difference in the cell extracts between the six sites.

C) Protein synthesis by dermal fibroblasts with the addition of FGFb

The protein levels for the cells listed in section 2.3.1. and treated with FGFb (10ng/ml) are shown in Figure 7.11c. The amount of protein released into the medium from the granulation tissue-derived cells was significantly lower than the normal cells ($p<0.05$). Figure 7.12c shows that the level of protein in the cell extracts of the granulation tissue-derived cells was significantly lower than the normal cells ($p<0.005$) and was significantly lower than the sarcoid cells ($p<0.001$). The sarcoid-derived cells were however, significantly higher than the normal cells ($p<0.05$).

There was no significant difference seen between the protein content of the six sites of the horse in the cell supernatants (Figure 7.13c) or cell extracts (Figure 7.14C).

D) Protein synthesis of dermal fibroblasts with TGFβ treatment

The amount of protein released into the medium of the granulation tissue-derived cells was significantly lower than the normal cells ($p<0.05$), shown in Figure 7.11d. Likewise, a decrease in protein was recorded in the granulation tissue-derived cell extracts ($p<0.02$) and the sarcoid-derived cell extracts ($p<0.05$) when they were compared to the normal cell extracts (Figure 7.12d).

As with the other growth factors there was no significant difference between the six sites of the horse in the cell supernatants (Figure 7.13d) or in the cell extracts (Figure 7.14d).

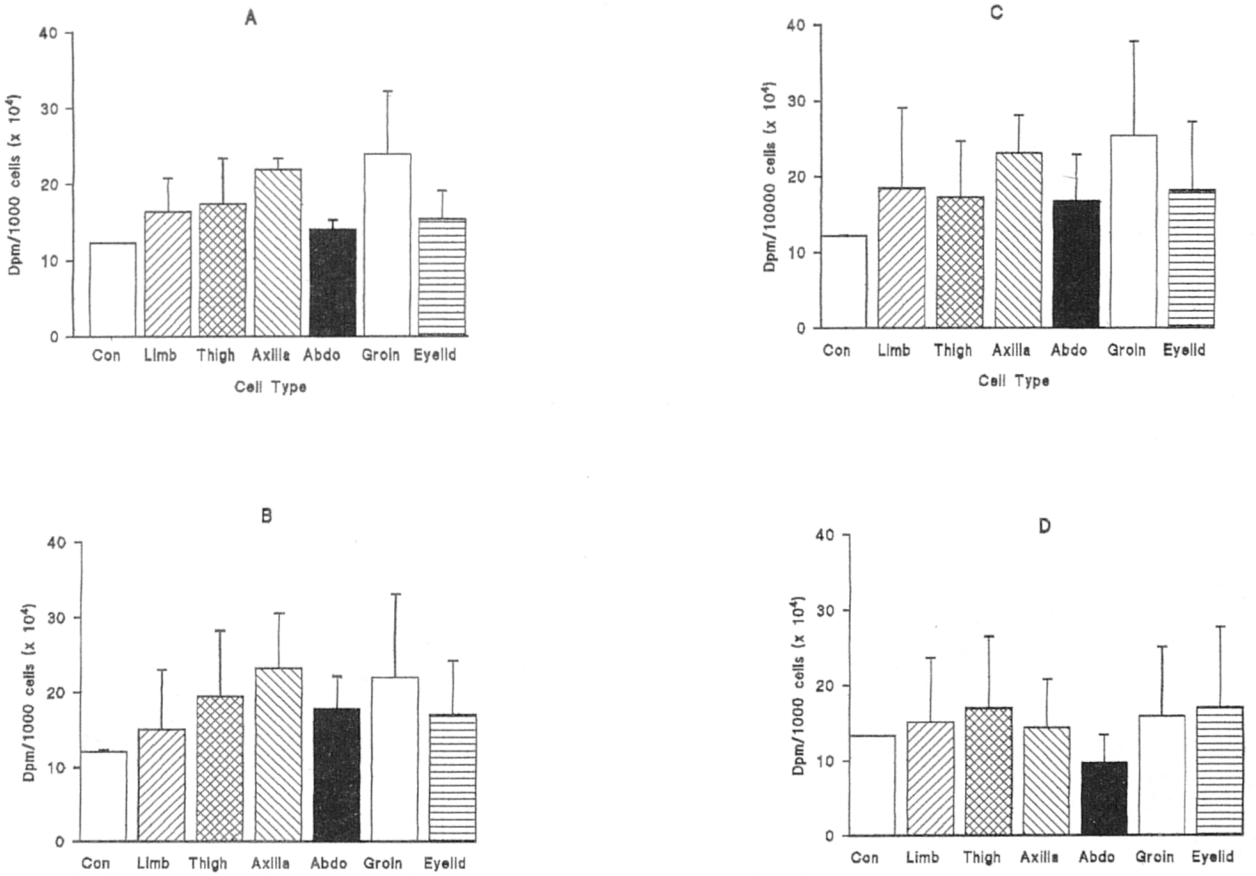


Figure 7.13 Incorporation of [3H] proline into the total protein of cell supernatants from different sites of the horse. Control cells (□); limb fibroblasts (▨); thigh (▩); axilla (▧); abdomen (■); groin (□); eyelid (▤); n=3.

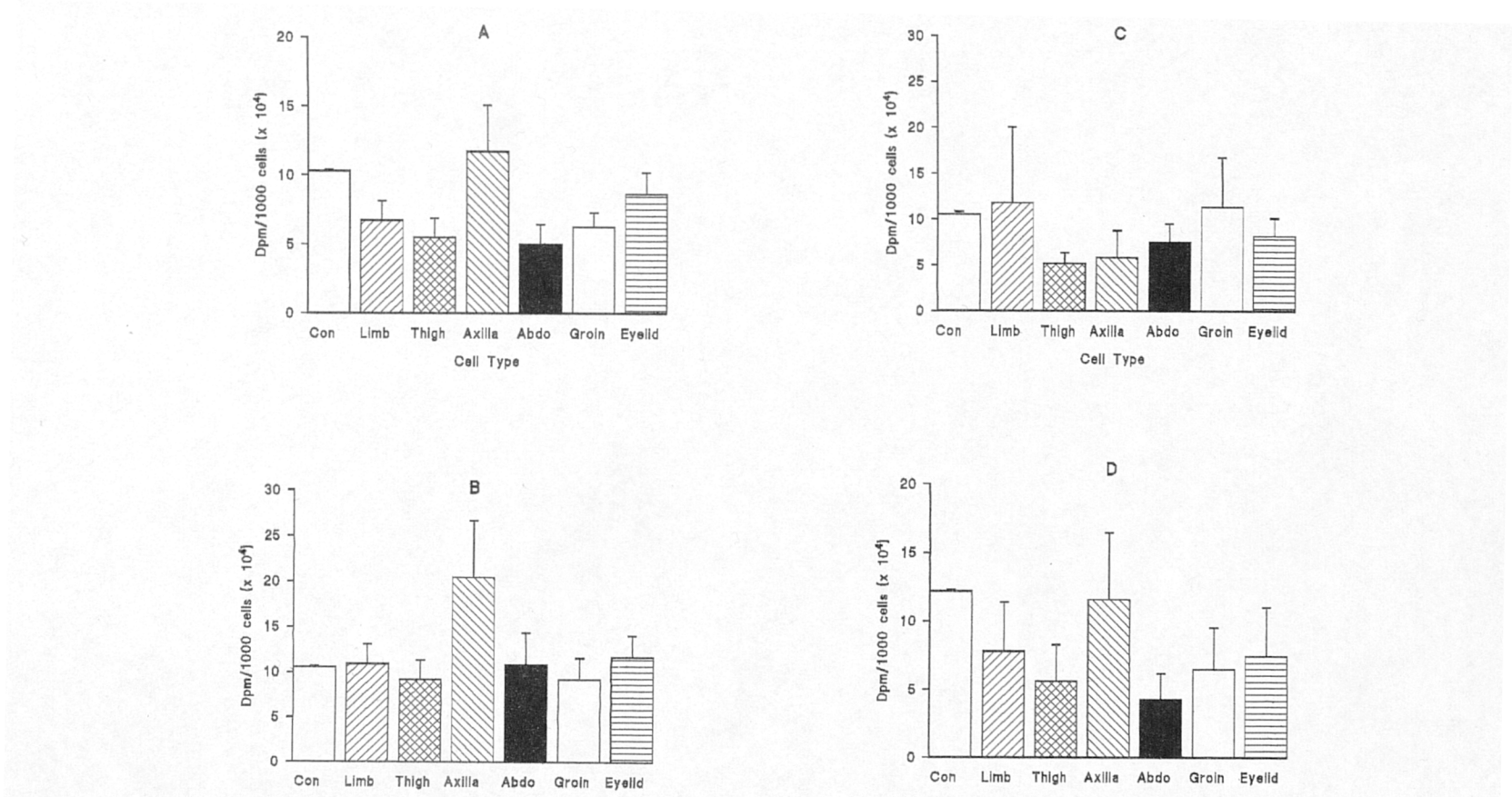


Figure 7.14 Incorporation of [3H] proline into the total protein of cell supernatantsfrom different sites of the horse. Control cells (□); limb fibroblasts (▨); thigh (▩); axilla (▧); abdomen (■); groin (□); eyelid (▤); n=3.

7.3.9 Effect of growth factors on collagen biosynthesis

A) Synthesis of collagenous and non-collagenous proteins after treatment with EGF

EGF had an inhibitory effect on collagen synthesis in the dermal fibroblast cultures. Table 7.1 shows the incorporation of [^{3}H]-proline into collagenous and non-collagenous proteins by normal, granulation tissue and sarcoid-derived cells. The collagenous protein was significantly higher in the cell supernatants of the sarcoid-derived cells than the normal cells ($p<0.0005$) and the granulation tissue-derived cells ($p<0.002$), shown in Figure 7.15a. There was no significant difference between the non-collagenous protein in the three cell types. Figure 7.16a, shows the collagenous and non-collagenous proteins of the cell extracts with the addition of EGF to the medium. The non-collagenous protein content of the normal cell extract was significantly higher than the granulation tissue cell extracts ($p<0.01$).

Collagenous and non-collagenous proteins were determined in the six sites previously mentioned. Figure 7.17a shows the levels of these proteins. The collagenous protein in the limb cell supernatants was significantly lower than in the thigh supernatants ($p<0.05$). Likewise, the eyelid cell supernatants contained significantly higher collagenous protein than the limb ($P<0.02$). In the cell extracts however (Figure 7.18a), there was no significant difference between any of the other five sites and the limb in collagenous or non-collagenous proteins.

Table 7.1 Collagen produced after treatment with EGF

Incorporation of [^{3}H]-proline into collagenous and non-collagenous protein by normal, granulation tissue and sarcoid-derived fibroblasts cultured for 24 hours with the addition of EGF (10ng/ml).

Incorporation of [^{3}H]-proline (dpm x $10^3/10^4$ cells)
Distribution

Cell supernatants	Total	CP	NCP	% CP
Control	9.9 ± 0.7	4.1 ± 0.2	5.8 ± 0.2	11.6
Normal	4.1 ± 0.6	1.1 ± 0.1	3.0 ± 0.5	8.7
Granulation	2.9 ± 0.5	0.6 ± 0.2	2.3 ± 0.3	4.5
Sarcoid	5.5 ± 0.7	3.2 ± 0.3	2.3 ± 0.4	21.3
Cell extracts				
Control	8.5 ± 0.9	1.3 ± 0.2	7.2 ± 1.2	3.28
Normal	2.7 ± 0.4	0.9 ± 0.2	1.9 ± 0.4	9.66
Granulation	1.1 ± 0.3	0.4 ± 0.1	0.7 ± 0.2	9.76
Sarcoid	4.9 ± 1.6	1.4 ± 0.4	3.5 ± 1.3	8.95

Each value is the mean of 3 separate incubations ± SEM. NCP, non-collagenous protein; CP collagenase-digestible protein. Control n=3; Normal n=3; Granulation n=4; Sarcoid n=4.

B) Synthesis of collagenous and non-collagenous proteins with FGFa treatment

Cell cultures from normal, granulation tissue and sarcoid-derived fibroblasts were treated with FGFa (10ng/ml). The collagenous and non-collagenous proteins were

calculated as described above (Table 7.2). There appeared to be no significant changes in the cell supernatants or extracts with FGFa treatment.

Table 7.2 Collagen produced after treatment with FGFa

Incorporation of [^{3}H]-proline into collagenous and non-collagenous protein by normal, granulation tissue and sarcoid-derived fibroblasts cultured for 24 hours with the addition of FGFa (10ng/ml).

Incorporation of [^{3}H]-proline (dpm x $10^3/10^4$ cells)
Distribution

Cell supernatants	**Total**	**CP**	**NCP**	**% CP**
Control	5.9 ± 0.6	1.1 ± 0.2	5.2 ± 0.3	3.9
Normal	5.8 ± 0.6	2.0 ± 0.3	4.0 ± 0.4	8.7
Granulation	3.0 ± 0.3	0.6 ± 0.2	2.4 ± 0.3	4.6
Sarcoid	5.1 ± 1.4	0.8 ± 0.3	4.3 ± 1.1	3.0
Cell extracts				
Control	6.1 ± 0.9	2.2 ± 0.3	3.9 ± 0.9	9.5
Normal	5.3 ± 0.7	1.5 ± 0.3	3.8 ± 0.7	11.3
Granulation	1.8 ± 0.5	0.8 ± 0.4	1.0 ± 0.1	11.7
Sarcoid	8.8 ± 2.9	1.9 ± 0.7	7.0 ± 2.7	5.3

Each value is the mean of 3 separate incubations ± SEM. NCP, non-collagenous protein; CP collagenase-digestible protein. Control n=3; Normal n=3; Granulation n=4; Sarcoid n=4.

However, the levels of collagenous protein in the normal cell supernatants was significantly higher than the granulation tissue ($p<0.005$) and sarcoid-derived cells ($p<0.05$) as shown in Figure 7.15b. The non-collagenous proteins in the normal cells supernatants were also significantly higher than the granulation tissue derived cells ($p<0.005$). In Figure 7.16b it can be seen that there was no significant difference between any of the cell extracts in their collagenous protein content. There was however, a significantly higher amount of non-collagenous protein in the normal cell extracts when compared to the granulation tissue ($p<0.002$). Figure 7.17b and Figure 7.18b respectively, show the collagenous and non-collagenous proteins in the cell supernatants and extracts of the six different sites treated with FGFa. There was no difference found between the cell types in either the cell supernatant or extract content of collagenous or non-collagenous protein.

C) Synthesis of collagenous and non-collagenous proteins with FGFb treatment

Labelled cell cultures were incubated with FGFb as described in the methods section and the collagenous and non-collagenous proteins were analysed as described above. The addition of FGFb to the cell cultures inhibited the synthesis of collagenous and non-collagenous proteins in the cell supernatants but had little effect on the cell extracts (Table 7.3). The collagenous and non-collagenous proteins from the three cell supernatants are represented in Figure 7.15c. The collagenous protein was significantly higher in the normal cell supernatants ($p<0.005$), when compared to the sarcoid supernatants. There was however, no difference between the cell extracts in collagenous or non-collagenous protein content Figure 7.16c.

Cell supernatants from the five sites were compared to the limb in their

collagenous and non-collagenous protein content (Figure 7.17c). Collagenous proteins in the eyelid were significantly higher than in the limb ($p<0.005$). The cell supernatants from the thigh and eyelid had significantly higher levels of non-collagenous protein ($p<0.05$) than the limb.

Table 7.3 Collagen produced after treatment with FGFb

Incorporation of [^{3}H]-proline into collagenous and non-collagenous protein by normal, granulation tissue and sarcoid-derived fibroblasts cultured for 24 hours with the addition of FGFb (10ng/ml).

Incorporation of [^{3}H]-proline (dpm x $10^3/10^4$ cells)
Distribution

Cell supernatants	**Total**	**CP**	**NCP**	**% CP**
Control	11.1 ± 2.2	4.5 ± 0.5	6.6 ± 0.8	11.2
Normal	3.7 ± 0.4	1.0 ± 0.3	2.7 ± 0.4	8.3
Granulation	2.4 ± 0.3	1.0 ± 0.1	1.4 ± 0.2	12.2
Sarcoid	8.8 ± 2.5	5.1 ± 2.1	3.7 ± 1.1	19.0
Cell extracts				
Control	5.0 ± 0.6	3.0 ± 0.4	2.0 ± 0.2	21.6
Normal	6.1 ± 0.7	3.6 ± 0.3	2.5 ± 0.4	5.5
Granulation	4.4 ± 3.1	2.0 ± 1.6	2.4 ± 1.4	8.6
Sarcoid	7.2 ± 4.6	2.1 ± 1.6	5.1 ± 2.9	5.3

Each value is the mean of 3 separate incubations ± SEM. NCP, non-collagenous protein; CP collagenase-digestible protein. Control n=3; Normal n=3; Granulation n=4; Sarcoid n=4.

The axilla also had significantly higher levels of non-collagenous protein than the limb ($p<0.01$). In the cell extracts (Figure 7.18c), the abdomen had significantly higher levels of collagenous and non-collagenous protein ($p<0.05$) than the limb.

D) Synthesis of collagenous and non-collagenous proteins with TGFβ treatment

Table 7.4 demonstrates the distribution of collagenous and non-collagenous proteins in normal granulation tissue and sarcoid-derived cell supernatants and extracts with TGFβ treatment, as described in methods.

The data in Figure 7.15d and 7.16d respectively represents the amounts of collagenous and non-collagenous proteins present in cell supernatants and extracts. The addition of TGFβ to the medium did not appear to alter the quantities of these proteins and there was no significant difference between the types of proteins in any of the cell types.

There was however, a significant difference between the limb cell supernatants and the axilla ($p<0.02$), abdomen ($p<0.05$) and eyelid ($p<0.01$) in the collagenous proteins (Figure 7.17d). Also, a significant difference ($p<0.005$) was observed between the limb and abdomen and the limb and groin. Figure 7.18d shows a significant difference between the limb and the eyelid cell extracts ($p<0.05$) in the non-collagenous protein content.

Table 7.4 Collagen produced after treatment with TGFβ

Incorporation of [^{3}H]-proline into collagenous and non-collagenous protein by normal, granulation tissue and sarcoid-derived fibroblasts cultured for 24 hours with the addition of TGFβ (10ng/ml).

Incorporation of [^{3}H]-proline (dpm x $10^3/10^4$ cells)
Distribution

Cell supernatants	Total	CP	NCP	% CP
Control	2.3 ± 1.2	0.4 ± 0.1	1.9 ± 0.2	3.5
Normal	1.5 ± 0.3	0.5 ± 0.1	1.0 ± 0.2	12.2
Granulation	2.8 ± 0.5	1.3 ± 0.7	1.5 ± 0.2	14.3
Sarcoid	4.0 ± 2.8	2.5 ± 2.1	1.5 ± 0.4	7.8
Cell extracts				
Control	1.5 ± 0.7	0.2 ± 0.4	1.3 ± 0.3	3.0
Normal	2.1 ± 0.7	1.1 ± 0.6	1.0 ± 0.4	18.7
Granulation	2.5 ± 1.4	0.8 ± 0.5	1.7 ± 0.8	7.4
Sarcoid	0.8 ± 0.3	0.2 ± 0.1	0.6 ± 0.3	6.3

Each value is the mean of 3 separate incubations ± SEM. NCP, non-collagenous protein; CP collagenase-digestible protein. Control n=3; Normal n=3; Granulation n=4; Sarcoid n=4.

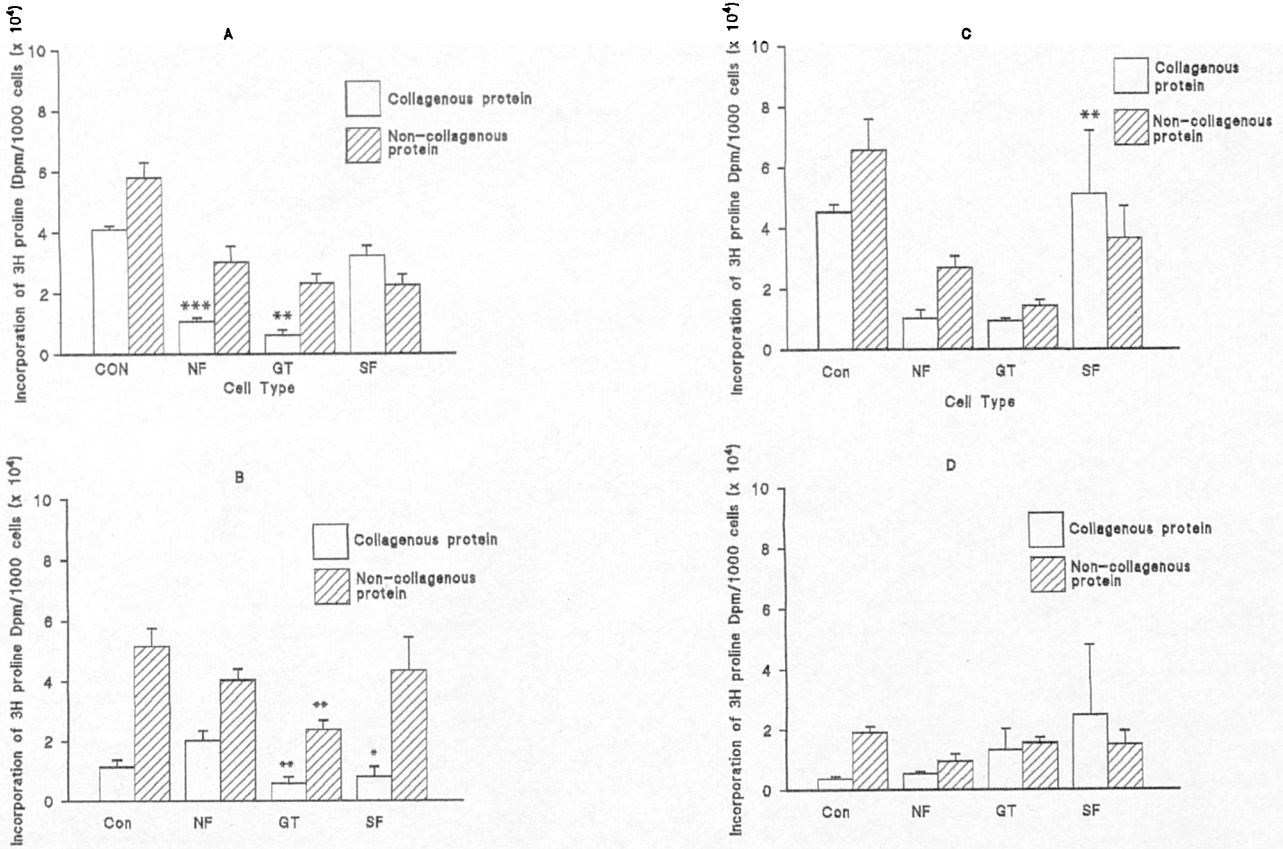


Figure 7.15 Synthesis of collagenous and non-collagenous proteins in cell supernatants of normal, granulation and sarcoid-derived fibroblasts after treatment with growth factors. Control (▨); n=3; normal fibroblasts (▨); n=3; granulation tissue-derived fibroblasts (▨); n=4; sarcoid fibroblasts (▨); n=4. A: treated with EGF (10ng/ml); B: FGFa (10ng/ml); C: FGFb (10ng/ml); D: TGFβ (1ng/ml). Values significantly different to normal and sarcoid cells *** $P<0.005$; sarcoid and granulation tissue-derived cells ** $P<0.002$ treated with EGF. Values significantly different to normal and granulation tissue-derived cells *** $P<0.005$; normal and sarcoid-derived cells * $P<0.05$ treated with FGFa. Values significantly different to normal and sarcoid-derived cells *** $P<0.005$ treated with FGFb in collagenous proteins.

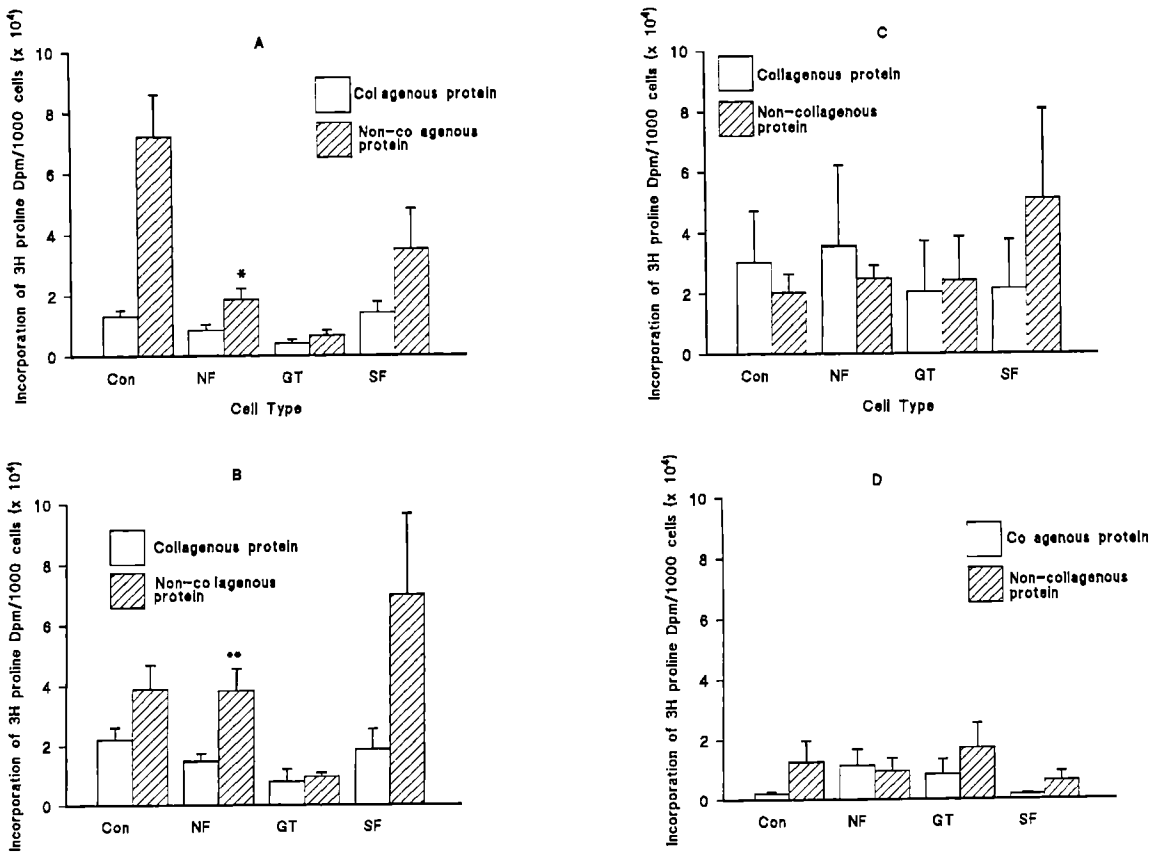


Figure 7.16 Synthesis of collagenous and non-collagenous proteins in cell extracts of normal, granulation and sarcoid-derived fibroblasts after treatment with growth factors. Control (☐); n=3; normal fibroblasts (☐); n=3; granulation tissue-derived fibroblasts (☐); n=4; sarcoid fibroblasts (☐); n=4. A: treated with EGF (10ng/ml); B: FGFa (10ng/ml); C: FGFb (10ng/ml); D: TGFβ (1ng/ml). Values significantly different to normal and granulation tissue-derived cells * $P<0.01$ treated with EGF. Values significantly different to normal and granulation tissue-derived cells ** $P<0.002$ treated with FGFa in non-collagenous proteins.

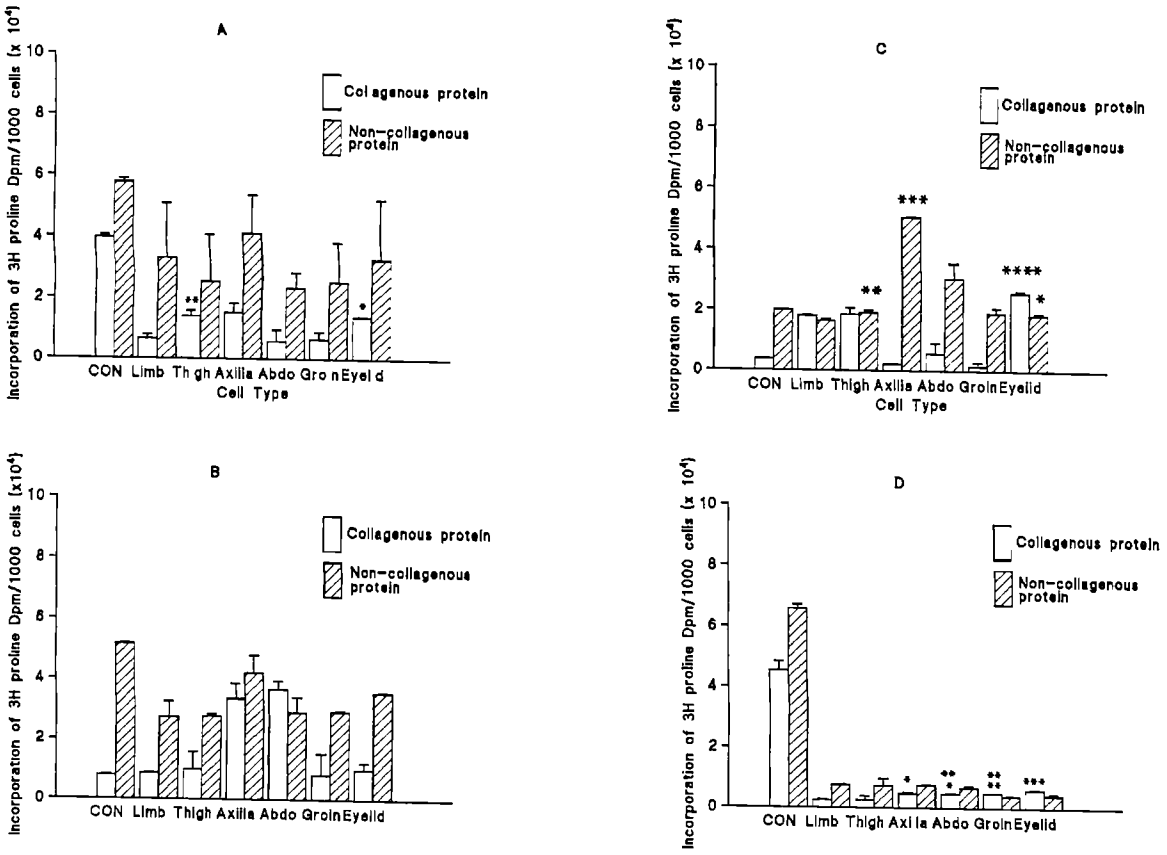


Figure 7.17 Synthesis of collagenous and non-collagenous proteins in cell supernatants in cells cultured from different sites in the horse after treatment with growth factors. Control (☐▨); limb (☐▨); thigh (☐▨); axilla (☐▨); abdomen (☐▨); groin (☐▨); eyelid (☐▨). A: treated with EGF (10ng/ml); B: FGFa (10ng/ml); C: FGFb (10ng/ml); D: TGFβ (1ng/ml). Values significantly different to limb and eyelid * $P<0.02$; limb and thigh ** $P<0.05$ treated with EGF. Values significantly different to limb and eyelid * $P<0.02$; limb and thigh ** $P<0.05$ treated with FGFa. Values significantly different to limb and eyelid **** $P<0.005$ in collagenous protein; limb and thigh, eyelid ** $P<0.05$; limb and axilla *** $P<0.01$ in non-collagenous proteins treated with FGFb. Values significantly different to limb and groin, abdomen **** $P<0.005$; limb and axilla * $P<0.02$; limb and eyelid *** $P<0.01$ treated with TGFβ in collagenous protein.

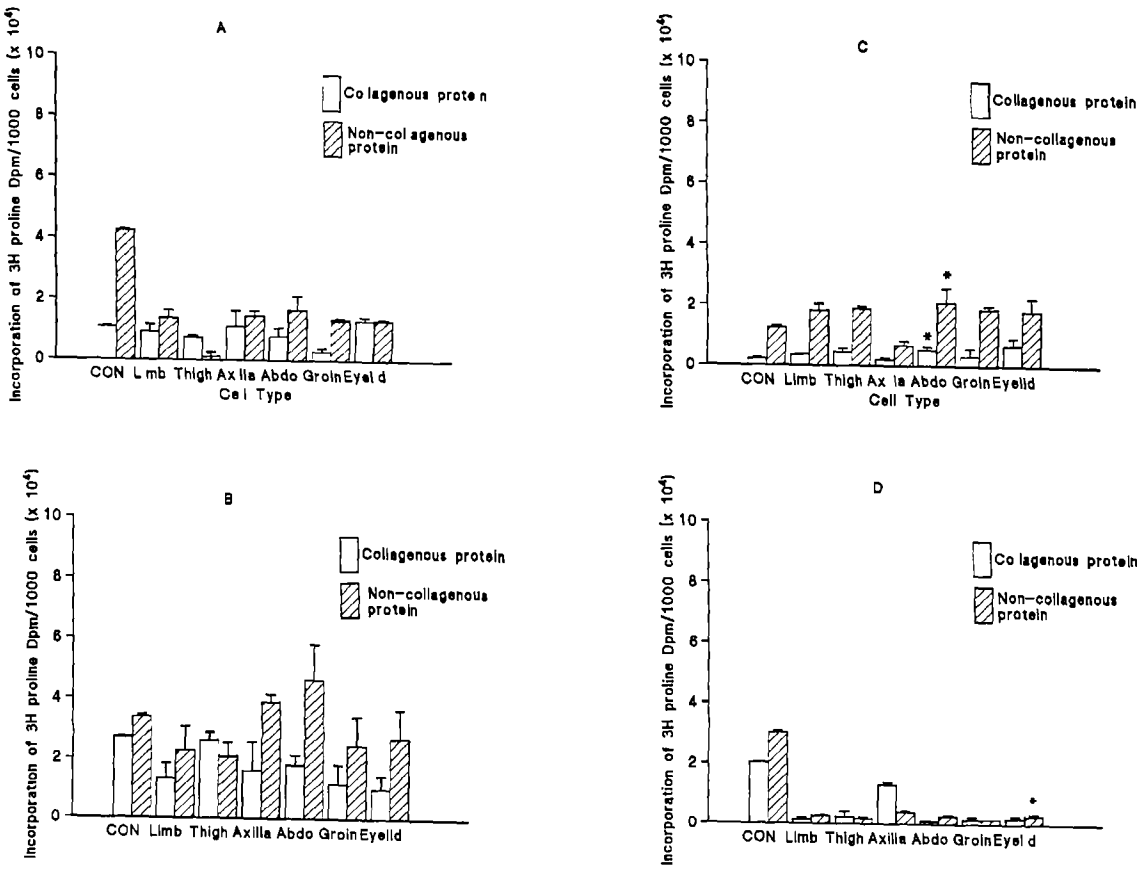


Figure 7.18 Synthesis of collagenous and non-collagenous proteins in cell extracts in cells cultured from different sites in the horse after treatment with growth factors. Control (□▨); limb (□▨); thigh (□▨); axilla (□▨); abdomen (□▨); groin (□▨); eyelid (□▨). A: treated with EGF (10ng/ml); B: FGFa (10ng/ml); C: FGFb (10ng/ml); D: TGFβ (1ng/ml). Values significantly different to limb and abdomen* $P<0.05$ in collagenous and non-collagenous protein treated with FGFb. Values significantly different to limb and eyelid * $P<0.05$ treated with TGFβ in non-collagenous protein.

7.4 DISCUSSION

The results from this study suggest that the problems associated with chronic equine wound healing and sarcoid formation are localised events. The fact that TGFβ levels were raised in wound fluids and granulation tissue cell supernatants but not PLA MA samples supports this hypothesis. More type I collagen was secreted into the supernatants of granulation tissue-derived cells than normal or sarcoid cells. This suggests that these granulation tissue-derived cells are producing excess type I collagen. Type I collagen is the most abundant protein component of granulation tissue (Kanzler *et al.*, 1986). There were also large quantities in wound fluids which suggests that there are large numbers of fibroblasts present within the wound or that the type I collagen synthesis by these cells has been upregulated in some way. The PICP present in is probably derived from the continuous turnover of skeletal collagen. In wound fluid the PICP concentration varies between two and a thousand times higher than that of serum (Haukipuro *et al.*, 1991).

Care was taken to ensure that the collagen assays were performed identically each time using the same number of cell passages, proliferative state of the cells, radioactive labelling time, protein degradation, specificity of the antibodies and enzymes used in the assays, culture conditions and experimental design. Low passage cells were used in all of the experiments and by using confluent cultures, the problems of different proliferative states could be avoided although the saturation densities may have been different. A labelling time of 24 hours was thought to be sufficient time for the growth factors to influence the metabolic processes of the cells. It has been suggested that the presence of serum in cultures eliminates the possibility of protein degradation as it contains sufficient

quantities of inhibitory factors to prevent protease activity (Regan *et al.*, 1991). A radiolabelled hydroxyproline assay (Juva & Prockop, 1966), was initially performed but only 8 samples could be analysed simultaneously and the results obtained were often variable and therefore of doubtful value.

Clinical factors such as the anatomical origin of the biopsy, the stage of healing when the biopsy was taken and the general heterogeneity of the cells between horses and within the same horse may produce difference biosynthetic results. Fleischmajer et al, (1981) found that fibroblasts obtained from different layers of human skin differ in their synthetic activity. Papillary fibroblasts proliferated more rapidly than reticular cells (Harper & Grove, 1979), while the latter showed higher synthetic activities. Although it is likely that cells isolated from their physiological environment acquire replicative and metabolic properties different from the *in vivo* situation, studies with diseased human fibroblasts have shown that cells in culture retained some of their physiological phenotypes and responded in a similar manner to that *in vivo* (Le Roy, 1974; Diegelman *et al.*, 1979; Fleischmajer *et al.*, 1981).

In this study EGF inhibited protein and collagen synthesis in the granulation tissue-derived and sarcoid cells. Epidermal growth factor is chemotactic for fibroblasts and many other cells types (Westermark & Blomquist, 1980). Although EGF does not induce synthesis of mRNA for extracellular matrix proteins such as collagen, it increases the numbers of fibroblasts in wounds through chemotaxis and mitosis, resulting in more total collagen production. EGF is also known to stimulate collagenase activity (Chua *et al.*, 1985). This could explain why EGF appeared to inhibit the collagen synthesis. It could be that the growth factor was inducing collagenase activity thereby inducing

collagen breakdown.

Both FGF a&b inhibited protein synthesis in all of the cell types studied. FGFs stimulate proliferation in most major cell types involved in wound healing both *in vivo* and *in vitro* but the effects of FGFs on collagen synthesis depends on the cell type. In addition, FGFb induces cell migration, neovascularisation and the formation of granulation tissue in animal models (Cordon-Cardo *et al.*,1990). Basic FGF is thought to stimulate collagen synthesis (McGee *et al.*, 1988). In this study it had an overall inhibitory effect which could have been due to the growth factor being broken down by proteases. It did however, stimulate collagen synthesis in cells derived from the eyelid, axilla and abdomen. This may be due to the fact that the skin in these areas is thinner than in other sites such as the thigh and it may be necessary for the fibroblasts to encourage the re-epithelisation of the skin as older epithelium is sloughed off.

TGFβ had no effect on the protein and collagen synthesis of the cells under investigation. In different concentrations TGFβ can either stimulate or inhibit cellular proliferation and its effect may be modulated by the presence of other growth factors. TGFβ is known to stimulate collagen synthesis (Sporn *et al.*, 1986; Roberts *et al.*, 1986). Interestingly, as with the addition of FGFb, TGFβ stimulated an increase in collagen synthesis in cells cultured from the eyelid, axilla and abdomen. Granulation tissue-derived cells have many characteristics similar to keloid scars in humans. Babu *et al.*, (1992) have shown that keloid fibroblasts do not increase protein synthesis in response to TGFβ. Also, a proportion of fibroblasts from keloids have increased type I procollagen mRNA, rates of procollagen production, and decreased rates of collagen degradation compared with fibroblasts from control skin. These findings correlate with

the findings from this study where there was an increase in the release of type I procollagen into the granulation tissue-derived cell supernatants. A hypothesis that these fibroblasts are bathed in high concentrations of growth factors and cytokines during the early phase of wound healing remain hyperactive and therefore contribute to the formation of hypergranulation tissue is interesting. This concept of a fibroblast which becomes persistantly active in response to exogenous stimuli, and passes this capacity on to subsequent generations despite repeated replication has been suggested in scleroderma (Bordin *et al.*, 1984).

From these studies it can be concluded that the growth factors studied impaired the ability of the cells to synthesise collagen *in vitro*. These decreased levels of collagen are likely to be due to degradation by wound proteases (Wysocki *et al.*, 1993). It would be advantageous to investigate the role of metalloproteinases in wound fluids and cell supernatants.

Experiments monitoring the rate of protein synthesis *in vivo* are ethically unacceptable in our equine subjects, as the welfare of horses is our major concern. Therefore these studies have been carried out using material derived from them during the course of normal surgery or at post mortem.

CHAPTER EIGHT

The role of metalloproteinases in equine wound healing

8.1 Introduction

Synthesis, deposition and degradation of extracellular matrix proteins are essential components of wound healing. Normal tissue repair follows a sequence of inflammation, granulation tissue formation and tissue remodelling (Clark, 1993). This sequence is disrupted in chronic wounds, resulting in delayed or lack of repair. Wound tissue turnover is probably mediated by members of the matrix metalloproteinase (MMPs), a family of zinc dependant endopeptidases that includes collagenases, gelatinases and stromelysins (Docherty *et al.*, 1992). Metalloproteinases are secreted by cells as inactive zymogens. This group of activated MMPs are capable of degrading all major extracellular matrix components (Docherty *et al.*, 1992). Metalloproteinases are inhibited by specific cell-secreted proteins, tissue inhibitors of metalloproteinases (TIMPs), that bind the active sites of MMPs non-covalently, but with high affinity, resulting in an inactive, protease-inhibitor complex (Cawston *et al.*, 1993; Howard & Banda, 1991).

In chronic wounds the normal sequence of wound healing is disrupted which results in lack of tissue repair and it is possible that matrix metalloproteinases may play a key role. In the horse however, there is no data available on the role of MMPs in wound healing. Spatial and temporal control of protcolysis is of critical importance in wound healing. Both MMPs and TIMPs have been shown to be expressed by cells in the wound environment and specific cell types may express these proteins differentially. This seems to be the case in the remodelling of the cornea, where cell-specific and

temporal expression of the two gelatinases varies during wound healing (Matsubara *et al.*, 1991). Keratinocytes and fibroblasts have different roles in wound repair, keratinocytes producing significantly more collagenase and less TIMP than fibroblasts *in vivo* (Saarialho-Kere *et al.*, 1992). Similarly, gelatinase A (MMP-2) localizes to fibroblasts, whereas gelatinase B (MMP-9) localizes to epidermal and endothelial cells (Oikarinen *et al.*, 1993; Salo *et al.*, 1994). Wound fluid reflects some of the changes that occur in the wound environment during healing. High levels of fibronectin and vitronectin degradation products indicative of excess proteolytic activity, have been detected in chronic wound fluids (Wysocki & Grinnell, 1990; Grinnell *et al.*, 1992). This fluid was subsequently shown to contain elevated levels of gelatinase A (MMP-2) and B (MMP-9)(Wysocki *et al.*, 1993).

As there have been no studies undertaken to investigate the role of metalloproteinases in equine wound healing, this study investigated the distribution of gelatinases in PLASMA, cell supernatants and wound fluids from non-healing equine wounds and sarcoid cases. The results were compared with known molecular weights of human MMPs (Table 8.1).

Table 8.1 **Molecular weights of human metalloproteinases**

(Woessner, 1991)

MMP	Latent	Active	Modified
MMP-1	57 & 52	48 & 42	24
MMP-2	72	66	-
MMP-3	60 & 57	50 & 48	28
MMP-9	92	84	-
MMP-10	53	47	28

8.2 Methods

8.2.1 Source of tissues and culture of dermal fibroblasts

Dermal tissue was obtained from the horses previously described in Chapter 2 (section 2.3.1).

Tissue culture samples were collected from early passage cells, with and without treatment with exogenous growth factors as previously described (Chapter 2), aliquoted and stored at -20°C until required for analysis.

8.2.2 Collection of PLASMA and wound fluids

Peripheral blood was collected from the horses described in section 2.5.2. PLASMA samples were separated from the clotted red blood cells (Section 2.5.2) aliquoted and stored at -20°C until required for analysis.

Wound fluid was collected from dressings that had been applied to non-healing wounds (Chapter 2, section 2.5.2.). Wound fluids were then aliquoted and stored at -20°C until required for analysis.

8.2.3 Tissue samples

Tissue samples were collected and homogenised from the same horses as described in chapter 2, section 2.2.1.

8.2.4 Zymography

Metalloproteinases in serum, wound fluids, tissue homogenates and cell supernatants were analysed by substrate-gel zymography described in Chapter 2, section 2.6.4.

8.2.5 Treatment of samples

Section 2.6.5. describes how samples were treated prior to being separated on zymograms by SDS gel electrophoresis.

8.2.6 Preparation of resolving gel

The preparation of the resolving gels is described in Chapter 2, section 2.6.6.

8.2.7 Preparation of stacking gel

A 4% stacking gel stacking gel was prepared as described in Chapter 2,

section 2.6.7.

8.2.8 Loading of samples and running of gels

Once the stacking gel was set the samples were carefully loaded onto the gel as described in chapter 2, section 2.6.8. A set of high molecular weight markers were run on each gel (Novex) as described in Chapter 2, (section 2.2.5).

8.2.9 Treatment of markers

When the gel run was completed, the stacking gel was removed and the markers were treated as described in section 2.6.9.

8.2.10 Treatment of gels

The resolving gels were treated with Triton X-100 and stained as previously described in section 2.6.10. A standard curve was created and the Rf values were calculated as previously described (Chapter 2, section 2.2.5).

8.3 Results

The relative molecular mass of the enzymes was calculated by comparing the Rf value of the enzyme with those on the standard curve. The molecular weights calculated from the zymograms were compared with known molecular weights of the human MMPs (Table 8.1).

8.3.1 Analysis of fibroblast and epithelial cell supernatants

a) Serum free cell supernatants

The normal dermal fibroblasts showed very little gelatinase activity (Table 8.2). Plate 8.1a reveals only one faint band of gelatinoytic activity at an apparent molecular weight of 64 kD. An enzyme of this molecular weight is likely to be the latent form of MMP-2.

The granulation tissue-derived cell supernatants (Plate 8.1b) showed four bands with gelatinolyic activity. These were: *a band with intense activity at 94kD* which corresponds to the latent form of MMP-9 and a band of slighltly less intense activity at 84kD which corresponds to the active form of MMP-9. A much larger band with high activity at 64kD and a further much fainter band at 59kD which corresponds to the latent and active forms of MMP-2 respectively. Interestingly, in Case 10026 (described in Chapter 3), the cell supernatants from the upper (healing) part of the wound contained all of the bands described above but the cell supernatants from the lower (non-healing) part of the wound displayed only one band at 64kD which relates to the latent form of MMP-2.

The sarcoid-derived cell supernatants revealed no visible enzyme bands on the zymogram.

Zymograms of epithelial cell supernatants (Plate 8.1b) revealed four major enzyme band. One at a molecular weight of 225kD which is the dimer form of MMP-9. A band of 94kD which is the latent form of MMP-9 and a band of 59kD which relates to the active form of MMP-2.

Table 8.2 Molecular weights of MMPs from cell supernatants (kD)

Cell Type	Serum free	2.5% FCS	2.5% FCS EGF	2.5% FCS FGFa	2.5% FCS FGFb	2.5% FCS TGFβ
Normal fibroblast	64	64	94 64	94 64	94 64	94 64
Granulation tissue fibroblast	94 84 64 59	94 84 64	94 59	94 59	94 59	94 59
Sarcoid fibroblast	no visible bands detected	64	94 64	94 64	94 64	94 64
Epithelial Cells	225 94 64 59	225 94 84 64 59	-	-	-	-

b) Analysis of cell supernatants in media containing 2.5% FCS

The normal dermal fibroblasts revealed only one faint band (Plate 8.2) with a molecular weight of 64kD which appeared to correspond to the latent form of MMP-2.

The granulation tissue-derived cell supernatants (Plate 8.2) revealed MMPs of 94kD, 84kD and 64kD which related to the latent and active forms of MMP-9 and the latent form of MMP-2 respectively. Interestingly, the cell supernatants from the non-healing part of the wound in Case 10026, showed the same bands as the cell supernatants from the healing part of the wound and with the same intensity. This suggests that the FCS contains a substance that stimulated these cells to produce more MMPs and in greater quantity.

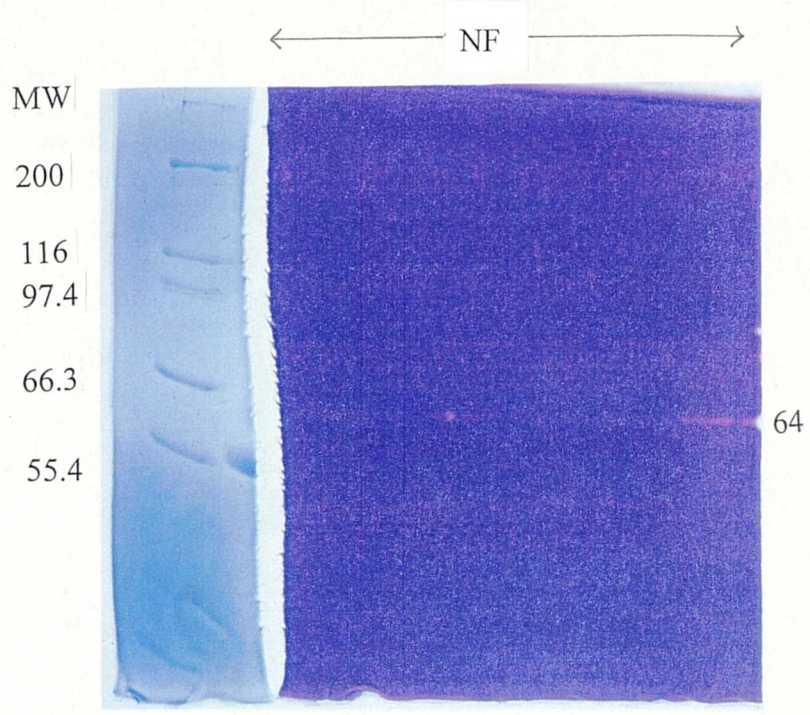


Plate 8.1a Zymogram of normal cell serum free supernatants

Legend: SF - Sarcoid derived cells; GT - granulation tissue derived cells; NF - normal dermal fibroblasts; E - Epithelial cells; FCS - Foetal calf serum;MW - Molecular weight markers

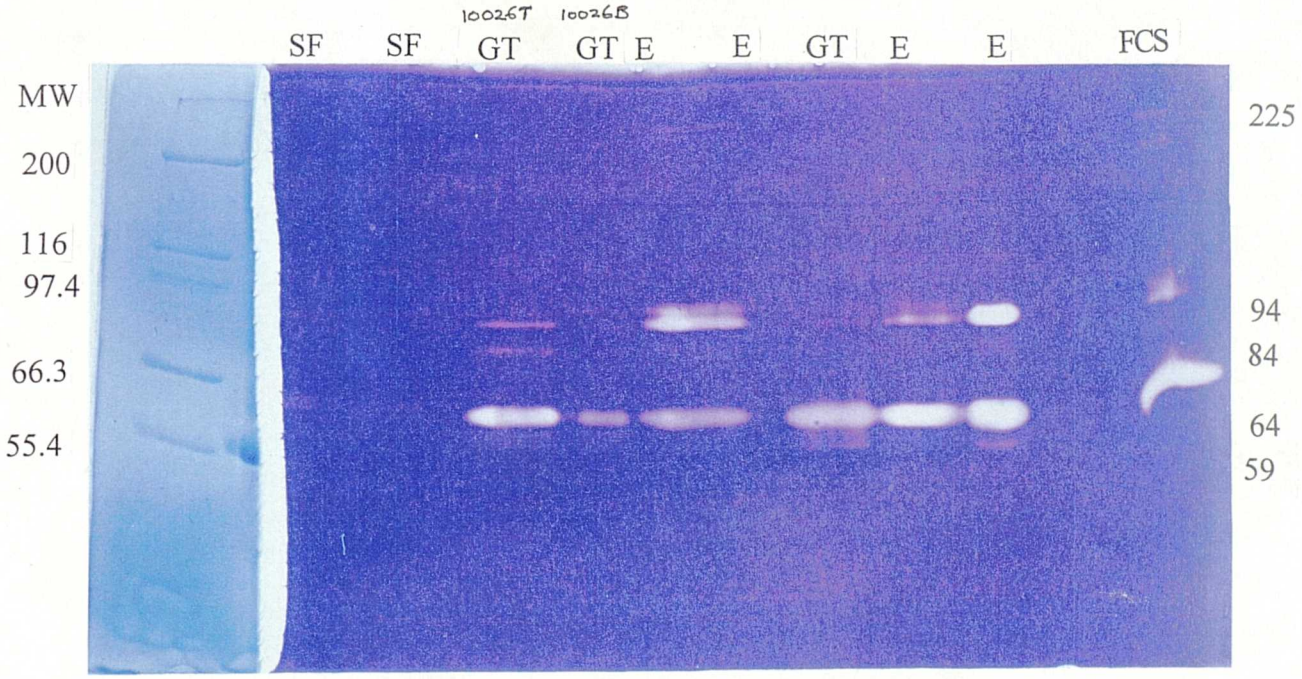


Figure 8.1b Zymogram of granulation, sarcoid and epithelial cell serum free supernatants

Legend: SF - Sarcoid derived cells; GT - granulation tissue derived cells; NF - normal dermal fibroblasts; E - Epithelial cells; FCS - Foetal calf serum; MW - Molecular weight markers; 2.5% - Control media containing 2.5% FCS.

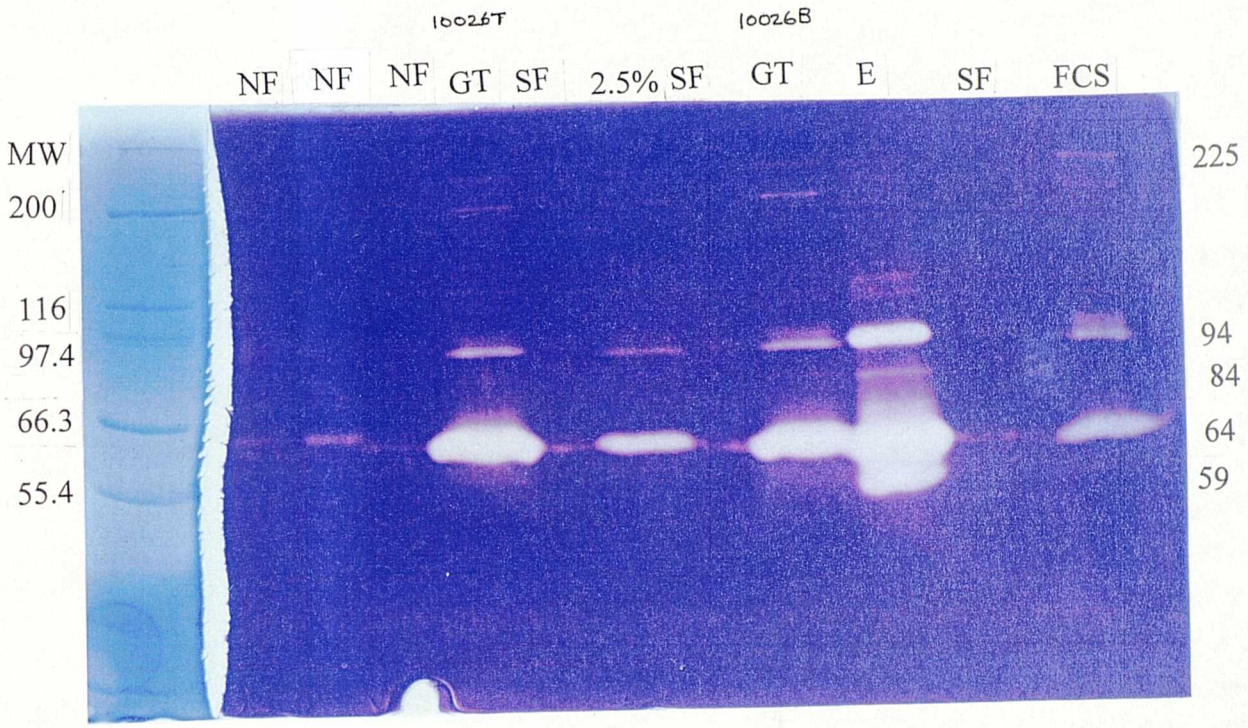


Plate 8.2 Zymogram of normal, granulation, sarcoid and epithelial cell supernatants with the addition of 2.5% FCS

The gelatinoytic activity in sarcoid-derived cells is shown in Plate 8.2. A faint band were located on the zymogram at 64kD. These correspond with human latent MMP-2. As with the non-healing wound (10026) the sarcoid cells were stimulated by the addition of FCS to the medium to produce the latent form of MMP-2.

The epithelial cell supernatants showed a greater amount of MMPs than with the serum free medium. The greater amount of activity appearing at 94kD, 64kD and 59kD. These bands correspond to the human latent MMP-9, latent MMP-2 and the active form of MMP-2. The dimer of MMP-9 was also present as a band at 225kD. Protein sequencing wound be necessary to make an accurate comparison with the human forms of these enzymes. This has recently been achieved in enzymes purified from neutrophils and synovial fibroblasts in equine joint disease (P.D. Clegg, personnal communication). It is evident that the FCS stimulated the production of the active form of MMP-2 in the sarcoid-derived cells as was the case with the granulation tissue-derived cells.

C) Analysis of cell supernatants with 2.5% FCS and growth factors

All of the cell types produced bands of digestion on the zymograms at 94kD with the addition of either EGF, FGFa, FGFb (10ng/ml) and TGFβ (1ng/ml) which compares with the latent form of MMP-9 (Table 8.2).

The normal cells when treated with EGF (10ng/ml, Figure 8.3), FGF a & b and TGFβ (1ng/ml) revealed bands of latent MMP-2 (64kD).

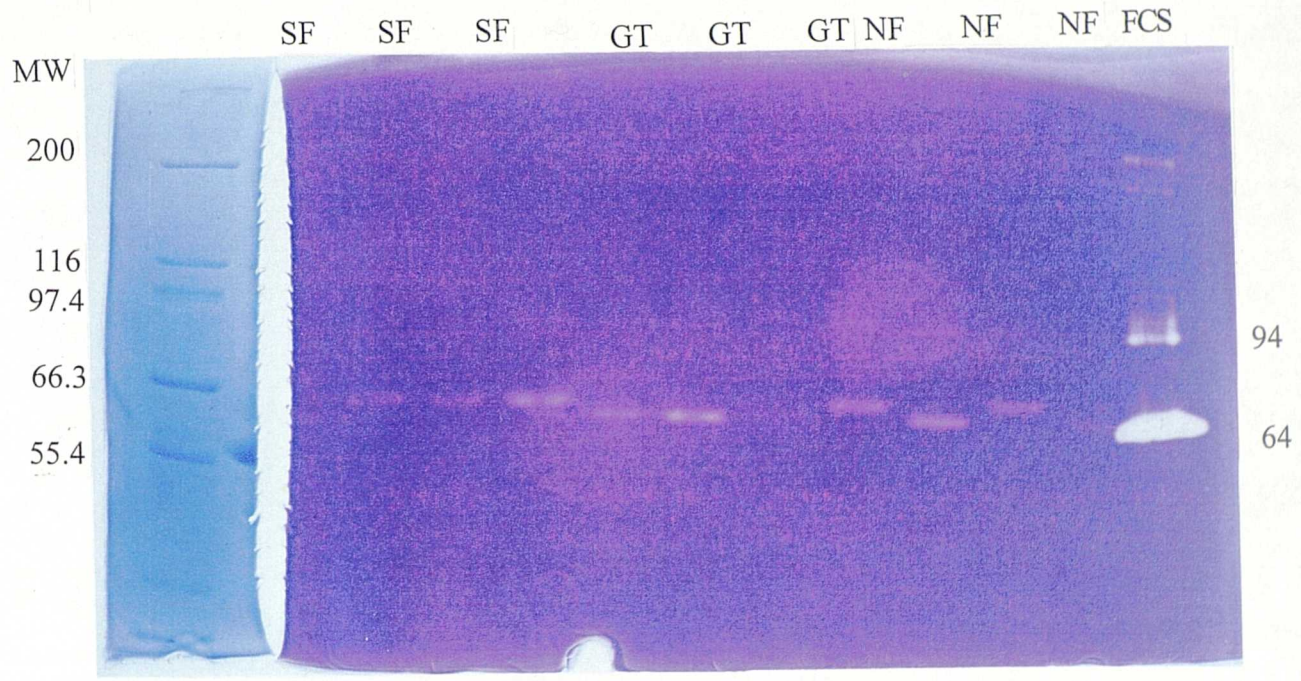


Figure 8.3 **Zymogram of normal, granulation, and sarcoid-derived cell supernatants with the addition of 2.5% FCS and EGF (10ng/ml)**

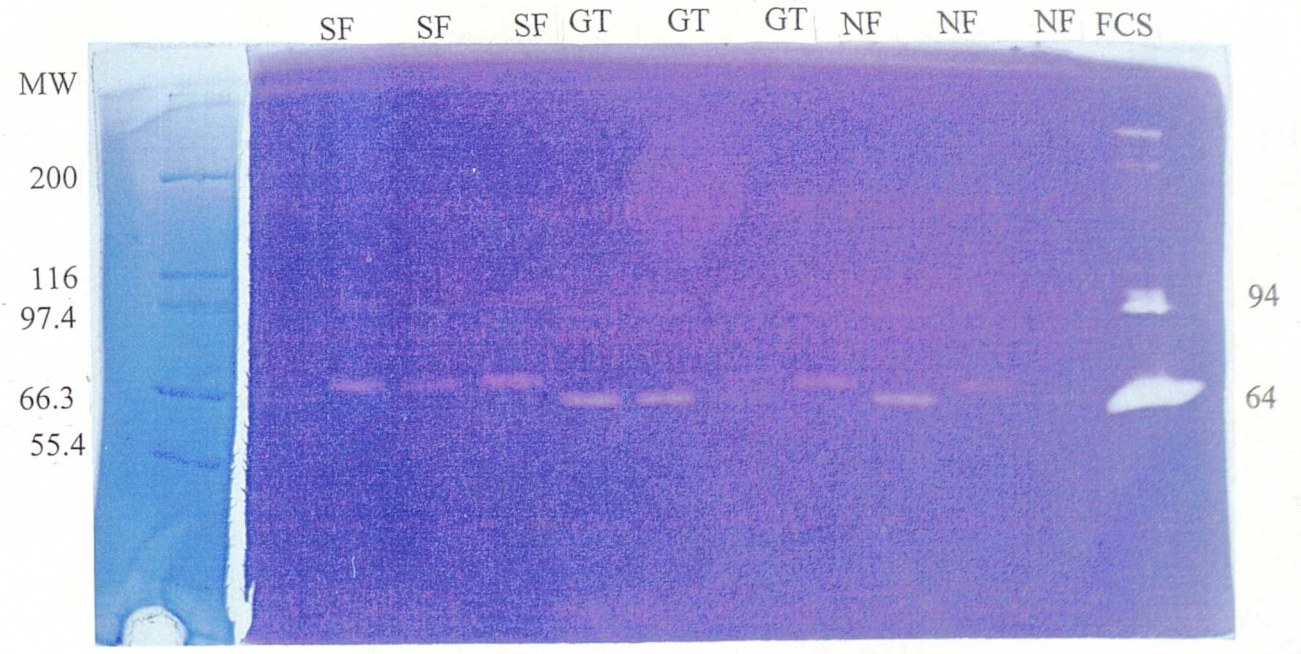


Figure 8.4 **Zymogram of normal, granulation and sarcoid-derived cell supernatants with the addition of 2.5% FCS and FGFa (10ng/ml)**

The granulation tissue-derived cells, stimulated with EGF, FGFa &b and TGFβ, produced an MMP of 59kD, similar to activated human MMP-2.

Stimulated sarcoid-derived cells produced latent forms of MMP-2 and 9. Fibroblast Growth Factor a & b treated cells produced a 94kD and a 64kD band which represents latent MMP-9 and latent MMP-2 respectively. Whereas, TGFβ (not shown) treated cells produced a 64kD band only, which corresponds with latent MMP-2. Treatment with EGF also resulted in the visualisation of an 64kD band which correlates with the latent form of MMP-2.

The granulation tissue-derived cells displayed brighter bands where the lower molecular weight MMPs were present on the zymograms, compared to the normal and sarcoid cells suggesting that these MMPs were more active or they were present in larger quantities.

8.3.2 Analysis of PLASMA

The types of MMPs in the PLASMA of normal horses, those with non-healing wounds and sarcoid lesions were analysed by zymography (Table 8.3). All of these horses displayed bands of 94kD and 64kD corresponding to the latent human form of MMP-9 and the latent form of MMP-2 (Figure 8.5). All of these bands on the zymograms had similar intensities suggesting that they had similar activities.

Table 8.3 Molecular weights (kD) of MMPs in PLASMA, wound fluid and tissue homogenates

Tissue type	PLASMA	Wound fluid	Tissue homogenates
Normal skin	94 64	——	64
Granulating wounds	94 64	225 94 84 64 59	94 64
Sarcoid	94 64	——	94 64

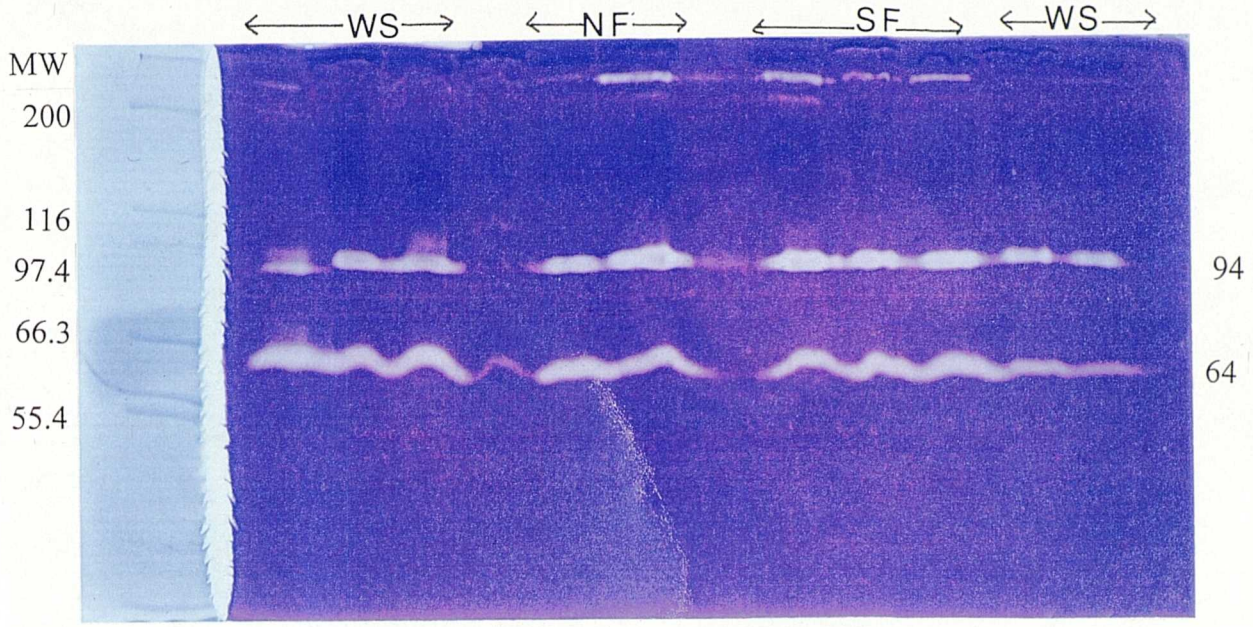


Figure 8.5 Zymogram of plasmasamples from normal, wounded and horses with sarcoids (1:10)

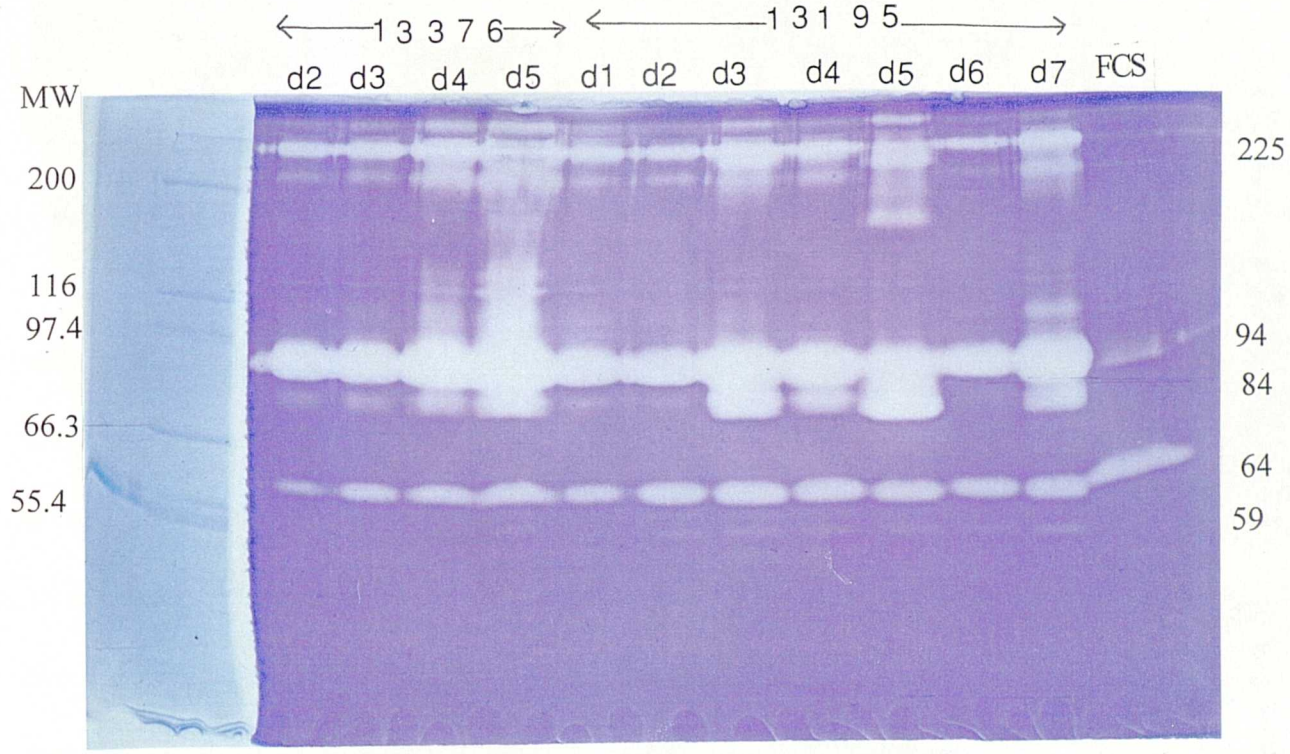


Figure 8.6 Zymogram of wound fluids from cases 13376 & 13195 (1:80)

8.3.3. Analysis of wound fluids

To determine the relative changes in gelatinase activity in wound fluids over time, samples were analysed by gelatin zymography (Table 8.3). Wound fluid from two different horses with non-healing wounds are shown in Plate 8.6. The two major proteinases migrated to where one would expect to find gelatinase A (64kD) and gelatinase B (94kD).

In case number 13376, the activity of gelatinase B increased on day 4 and increased further on day 5. Initially the molecular weight of the gelatinase B was approximately 94kD corresponding to the latent human MMP-9. However on days 4 and 5 the molecular weight was approximately 84kD which related to the active form of MMP-9. Likewise, in case number 13195 the same occured on days 3, 4, and 5 where the MMP-9 was activated. On day 6, the activity decreased but on day 7 increased again. This suggests that the inflammatory phase of wound healing is being switched off and then switched on again.

Figure 8.7 shows case number 12968. In lanes 2 and 3 wound fluid from a healing wound on the abdomen reveals a very faint band of 94kD which relates to the latent form of MMP-9. The activity in lanes 5-19 showed that the levels of gelatinase B and A were very active initially and decreased dramatically over time. In lane 13 the activity was beginning to increase. In lane 17, the activity was higher still and in lane 18 started to reduce. The active form of MMP-2 (59kD) was evident and more intense over time. These experiments have been repeated several times with similar results. It may be necessary to sequence these enzymes in order to make a direct comparison with the published human data.

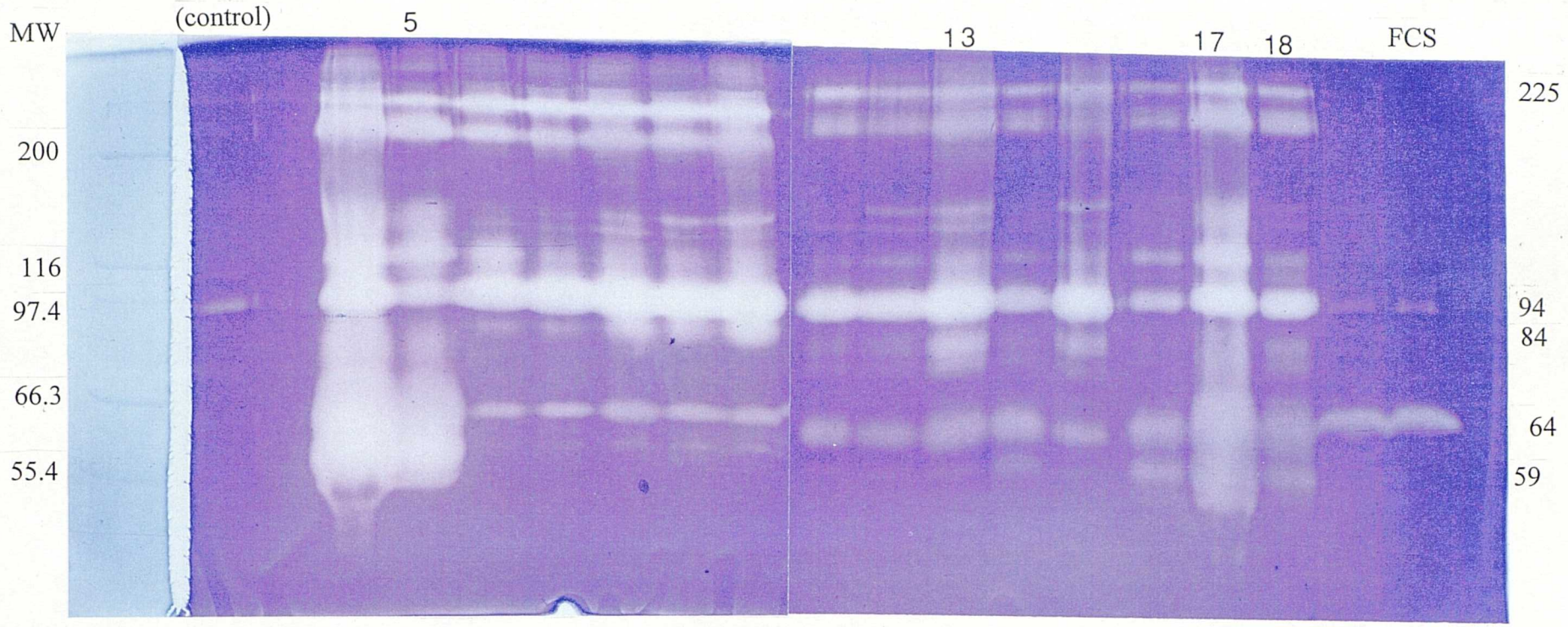


Figure 8.7 **Zymogram of wound fluid from case 12968 (1:100). Wound fluid samples were collected on consecutive days, up to day 18.**

8.3.4 Analysis of tissue homogenates

Gelatinase activity was analysed in tissue homogenates by gelatin zymography (Figure 8.8). The major bands of activity in the granulation tissue were 94kD (which corresponds to the latent form of MMP-9), 64kD (which correlates with the latent form of MMP-2).

The sarcoid homogenates revealed the same pattern of activity. A band of 94kD which corresponds to the latent form of MMP-9, a 64kD band which correlates with the latent form of MMP-2.

The normal tissue homogenates revealed only one band at 64kD which correlated to the latent form of MMP-2. These bands were however, much fainter in the normal tissue homogenates than the sarcoid and the granulation tissue homogenates.

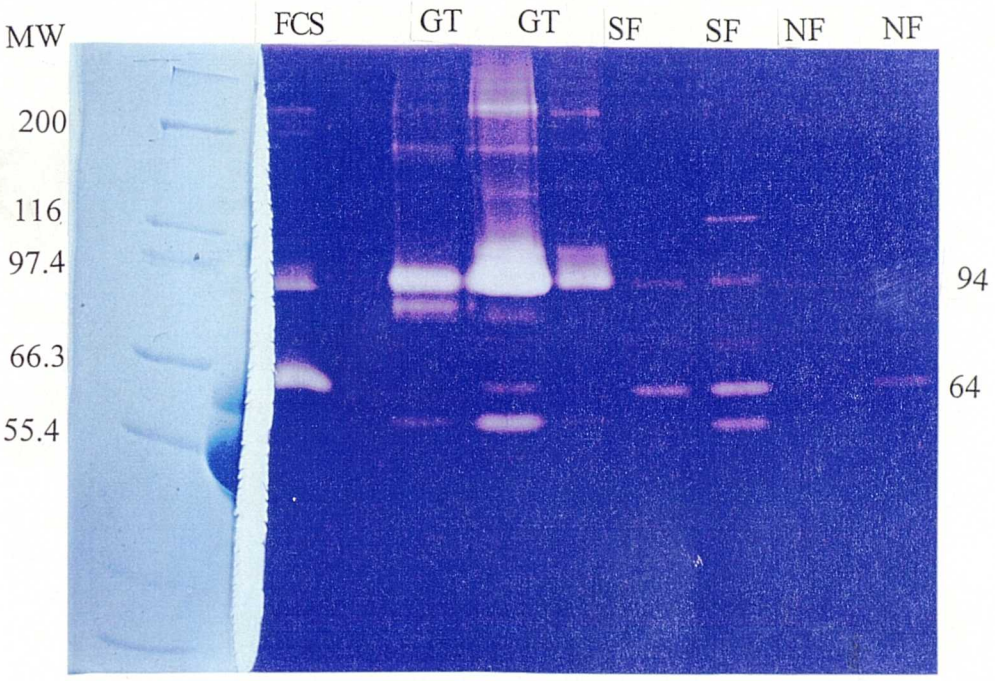


Figure 8.8 Zymogram of normal, granulation and sarcoid-derived tissue homogenates (1:10)

8.4 Discussion

Wound healing involves the controlled and co-ordinated synthesis and degradation of extracellular matrix components. It is likely that the balance between proteinase and inhibitor levels plays a critical role in the successful healing process. Any disruption of this balance may lead to unsuccessful healing. In this study the amounts of gelatinase MMP-2 and MMP-9 were analysed in cell supernatants, plasma, wound fluids and tissue homogenates. Although not accurately quantified clear differences were seen between tissues, in time and as a result of culture with growth factors.

The addition of serum to the medium of the cell cultures stimulated the release of latent MMP2 into the sarcoid cell supernatants. There was little effect on the normal and epithelial cell supernatants. It is possible that cytokines or growth factors within the serum added to the medium of the cells may have had either stimulatory or inhibitory effects.

Salo and co-workers, (1991) reported that epidermal cells are able to synthesise MMP-2 although in small quantities. Wound fibroblasts however from pigs (Agren, 1994), rats (Sakata *et al.*, 1989) and humans (Salo *et al.*, 1991) can continually synthesise MMP-2. Inflammatory cells do not produce MMP-2. The activity of MMP-2 may reflect the number of fibroblast in the wounds (Dyson *et al.*, 1988).

The addition of growth factors to the medium stimulated the synthesis of the active form of MMP-2 in the granulation tissue cell supernatants and the latent form of MMP-9 in the normal dermal cell supernatants. Many growth factors including TGFβ, IGF-1 and FGFb are known to be secreted in the latent form and activated by limited proteolysis (Folkman *et al.*, 1988; Wakefield *et al.*, 1988; Gonzalez *et al.*, 1990; Saksela

& Rifkin, 1990). When placed on type I collagen, keratinocytes produce three times more MMP-9 than when they migrate on type IV collagen (Sarret *et al.*, 1992a), experimental conditions which mimic the contact of keratinocytes with a raw wound surface and a basement membrane, respectively.

Wysocki *et al.*, 1993, showed that wound fluid from chronic wounds contained markedly elevated levels of several MMPs compared with serum. This study suggests that the same is occurring in the horse. This suggests that the synthesis and release of these MMPs is localised and not systemic. Therefore, the chronic wound environment represents a highly proteolytic environment. The 225kD located in the epithelial cell supernatants and wound fluids is probably the dimer form of MMP-9 which has been shown to dimerise when it is present in excess relative to TIMP. When enough TIMP is present, this dimerisation is inhibited. This dimerisation may be important in the control of activation as it can be activated by stromelysin, unlike the MMP-9/TIMP complex (Goldberg *et al.*, 1992).

The activity of gelatinase MMP-2 and 9 was significantly elevated in the first few days after wounding and later decreased over time. It is possible then that as elevated levels of MMP-2 and 9 are being synthesised to breakdown denatured collagen in early wound healing , in the later stages as these MMPs are less active.

The cellular sources of gelatinases in wound fluid are not yet known. It is possible that much of the gelatinase MMP-9 is derived from infiltrating neutrophils that are known to migrate rapidly into wound sites. These cells store gelatinase MMP-9 in granules (Kjeldsen *et al.*, 1994), and it is possible that a proportion of this proteinase present in early wound fluid samples is neutrophil derived. Previous studies have shown

that gelatinases can be derived from tissue near the wound site (Oikarinen *et al.*, 1993; Salo *et al.*, 1994). In this study, we have demonstrated that activated MMP-2 is being synthesised by normal skin albeit in very low levels compared to the sarcoid and granulation tissue. Both gelatinase MMP-2 and MMP-9 were found during re-epithelisation in a human suction-blister model (Oikarinen *et al.*, 1993) which is in agreement with our study of the granulation and sarcoid tissue homogenates. Oikarinen and co-workers (1993), found that the gelatinase MMP-2, which increased moderately within the first 24 hours post-wounding, was localised to fibroblasts, whereas gelatinase MMP-9, which increased roughly threefold within 24 hours, localised to the epidermis and endothelial cells.

This study has demonstrated that both active and latent forms of gelatinase MMP-2 and MMP-9 are present in wound fluids from chronic equine wounds. Wysocki *et al.*, (1993) identified similar gelatinase activity in wound fluids from human chronic leg ulcers. This could explain the presence of fibronectin and vitronectin degradation products found in chronic wound fluid (Wysocki & Grinnell, 1990; Grinnell *et al.*, 1992).

Chronic wounds express elevated levels of MMPs that decrease with time and further increased levels are seen later in the wound repair process which indicates a persistent inflammatory response. The realisation that chronic wounds are highly active tissues with a common, high proteolytic potential has important implications for the treatment of chronic wounds.

Zymography demonstrates the presence of metalloproteinases and their relative activities. MMPs are secreted as inactive zymogens, and activation requires loss of an N-terminal propeptide and thus a loss of molecular weight. Activation *in vivo* is poorly

understood although it is thought to involve the plasminogen activator/plasmin system and other metalloproteinases (Woessner 1991). Gelatin zymography allows measurement of both the zymogen and active MMP, as when the MMP is run on the gel it is activated and all inhibitors are removed from the molecule (Birkedal-Hansen and Taylor, 1982). A membrane bound activator of MMP-2 has recently been identified, known as membrane-type metalloproteinase (MT-MMP) and is possibly the only route of activation of MMP-2 (Nomura *et al.*, 1995; Strongin *et al.*, 1995). It would be of interest to analyse the active forms of these enzymes using an ELISA method recently developed in our laboratories and to quantify them. These enzymes could be sequenced thereby, enabling a direct comparison to human MMPs to be made. Further zymography studies could be undertaken to investigate stromelysin activity using casein as the substrate and collagenase activity using collagen as the substrate in the polyacrylamide gels.

CHAPTER NINE

General Discussion

9.1 Summary of findings

The work presented in this thesis was designed to investigate some of the factors that influence the character and rate of wound healing and sarcoid formation in the horse. This chapter will summarise the findings and discuss the important questions raised concerning these problems and the roles of growth factors and extracellular matrix molecules. The main findings of the research presented in this thesis are:

1. Pericapillary cuffs were identified around blood vessels localised in the wound area and at the wound margin. These cuffs were composed of not only fibrin but also laminin, fibronectin, collagen and endothelial cell adhesion molecules. There was a limited presence of fibronectin in the wound area but an abundance of type I collagen. There was a strong growth factor profile within the wounded tissue, TGFβ1 being most intense at the wound edge and TGFβ2 and PDGF around capillaries.

2. Biochemical analysis of the tissues revealed high levels of protein, hydroxyproline, type I procollagen (PICP) and TGFβ in the granulation tissue. The sarcoid tissue also had elevated levels of type I procollagen (PICP) and TGFβ when compared to the normal tissue but to a lesser degree.

3. Normal, granuation and sarcoid-derived cells were dependant on serum for proliferation and responed in a dose dependant manner. These cells differed in:

    a) Morphology of the cells

    b) Growth rate characteristics

    c) Quantities and type of collagen produced

    d) Protein synthesis

    e) Metalloproteinase profile

    f) Response to growth factors

4. TGFβ and type I collagen concentrations were similar in PLASMA from normal horses, horses with wounds and horses with sarcoids. In contrast, TGFβ and type I collagen was elevated in wound fluids, suggesting a localised event as opposed to a systemic one.

5. The metalloproteinase profile was unchanged in the PLASMA from the normal, wounded and sarcoid cases. However, a marked difference was recorded in the type of metalloproteinases in the granulation tissue and sarcoid homogenates compared to the normal tissue homogenates. The activity of gelatinase MMP-2 and MMP-9 in wound fluids significantly increased in the first few days post wounding and decreased over time.

9.2 What are the roles of extracellular matrix molecules and adhesion molecules in equine wound healing?

Fibronectin was present in the surrounding skin but less evident in the wound area. Fibronectin is known to have multiple functions in wound healing and is a major component of skin. It seems likely that a reduced deposition of fibronectin in the wound area may play a role in the impaired healing of chronic wounds. This deficiency of fibronectin in the wound may be associated with excessive degradation rather than poor synthesis. Wysocki and co-workers suggested that proteases are over expressed in chronic wounds and degradation fragments of fibronectin are abundant in wound fluids (Wysocki *et al.*, 1991 & 1993).

Collagen type I was located within the wound and type IV in the basement membranes of the tissues. Hydroxyproline levels were elevated in the granulation tissue homogenates and to a lesser extent in sarcoid tissues. Granulation tissue-derived fibroblasts synthesised and released greater amounts of hydroxyproline and type I collagen into the medium than either normal or sarcoid-derived cells. Type I collagen is known to be the most abundant protein component of granulation tissue (Kanzler, 1986). These findings suggest that the increased collagen content of the granulation tissue may be due to either an over abundance of fibroblasts or that collagen synthesis has been upregulated by these fibroblasts *in vivo*. The increase in fibroblasts numbers could be due to the presence of growth factors, known to be chemoattractants for fibroblasts at elevated levels. There was intensive staining for endothelial cell adhesion molecules in the wound area and in the granulation tissue-derived fibroblasts which suggests that angiogensis takes place within granulation tissue. It is known that endothelial cell adhesion molecules are expressed at sites of inflammation and injury. ICAM-1

expression is increased in severely diseased skin (Wilkinson *et al.*, 1993) and PECAM is known to be present around blood vessels in normal and inflammed skin (Albelda *et al.*, 1991).

9.3 What role do growth factors have on equine wound healing and sarcoid formation?

The skin biopsies showed intense staining for growth factors such as EGF, TGFβ, PDGF and FGF. It has been commonly thought that chronic wounds were deficient in growth factors. In this study however, we have shown that the chronic non-healing equine wound consists of a large number of cytokine synthesising inflammatory cells and fibroblasts. Immunocytochemistry does not distinguish between intact and partially degraded growth factor peptides or between active or inactive variants of the same growth factor. Growth factor receptor levels may be low or of a different receptor isoform type. Low levels of growth factor receptors may occur as a down regulation event in response to high levels of growth factors present or may be degraded by proteases. Normal human cells have aproximately 20,000 EGF receptors per cell (Gill *et al.*, 1987). High levels of TGFβ were detected in granulation tissue homogenates, cell supernatants and wound fluids. TGFβ is known to be synthesised by macrophages and other inflammatory cells, which suggests that the inflammatory phase of the healing wound was continuous hence the wound was unable to heal. Growth factors (EGF, FGF) inhibited proliferation of the granulation-tissue derived cells but TGFβ revealed a mild stimulatory effect. Loef and co-workers (1986) reported that TGFβ can increase the rate of cell proliferation in some fibroblast cell types via induction of a platelet derived growth factor-like protein, its predominant effect however, is to inhibit cell growth

(Moses *et al.*, 1985).

9.4 Regulation of matrix metalloproteinases

Gelatinases MMP-2 and MMP-9 were predominant in wound fluids and cell culture supernatants. High levels of these enzymes in wound fluids suggests that the newly synthesised extracellular matrix molecules in the wound may be degraded. It is necessary for a reasonable level of metalloproteinases to be present in the wound environment to assist in the debridement of damaged tissues. Many researchers have reported high levels of matrix metalloproteinases in wound fluid during early wound healing and a progressive decrease in activity over time. Gelatinase MMP-2 increased moderately in the first 24 hours post-wounding and was localised to fibroblasts, whereas gelatinase MMP-9 was localised to the epidermis and endothelial cells (Oikarinen *et al.*, 1993). The source of gelatinases in wound fluid is not known. It is possible that the gelatinase MMP-9 is derived from the granules of infiltrating neutrophils which are known to migrate rapidly into wound sites at an early stage (Kjeldsen *et al.*, 1994). In this study however, we found that the enzyme activity decreased after the first few days but there was a further increase later in the healing process. This suggests that the inflammatory response in the wound is not being switched off and the wound remains inflammed indefinitely. It suggests therefore that exuberant granulation tissue is formed and continues to proliferate if left untreated.

9.5 Future studies

The research studies in this thesis have indicated a number of possible areas of further work which would benefit our understanding of equine wound healing and sarcoid formation:

1. It would be useful to assess the quantities of fibronectin in granulation and sarcoid tissue biochemically. This would confim the low levels that were detected by immunocytochemistry. It would also be interesting to investigate the tissue architecture of the sarcoid tissue especially with reference to growth factor profiles and endothelial cell adhesion molecules by immunohistochemistry.

2. Further cell culture studies using collagen and fibronectin substrata to assess whether morphology, proliferation, protein and collagen synthesis are affected.

3. Further investigation into the role of stromelysin and collagenases in wound healing and sarcoid formation. These enzymes could be purified and characterised. The investigation of TIMPs and their role in wound healing would also be advantageous.

4. Characterisation of the epithelial cell lines and co-culture experiments with dermal fibroblasts to produce an *in vitro* wound model.

5. The use of epithelial cells grown on synthetic skin and applied to equine wounds may be benefical in equine wound healing. This would eliminate the requirement for skin grafting and result in a reduction of cost and trauma to the horse.

REFERENCES

Adams R., Calderwood-Mays M.B., Peyton L.C. (1988) Excision of cutaneous tumours in the horse using histologic guidance. Vet. Surg. 17: 241-245.

Agren M. S. (1994) Gelatinase activity during wound healing. Br. J. Dermatol. 131: 634-640.

Albelda S.M., Muller W.A., Buck C.A., Newman P.J. (1991) Molecular and cellular properties of PECAM-1 (endoCAM/CD31): A novel vascular cell-cell adhesion molecule. J. Cell Biol. 114: 1059-1068.

Ali I.U. & Hynes R.O. (1978) Effects of LETS glycoprotein on cell motility. Cell 14: 439-446.

AlitaloK., Hovi T., & Vaheri A.. (1980) Fibronectin is produced by human macrophages. J. Exp. Med.151: 602-612.

Angelos J.A., Marti E., Lazary S., Carmicheal L.E. (1991) Characterisation of BPV like DNA in equine sarcoids. Arch. Virol.119: 95-109.

Ansel J.C., Tiesman J.P., Olerud J.E., Krueger J.G., Krane J.F., Tara D.C., Shipley G.D., Gilbertson D., Usui M.L., Hart C.E. (1993) Human keratinocytes are major source of cutaneous platelet derived growth factor. J. Clin. Invest. 92: 671-678.

Antoniades H.N. (1981) Human platelet derived growth factor: Purification of PDGF I and PDGF II and separation of their reduced subunits. Proc. Natl. Acad. Sci. USA 78: 7314-7.

Antoniades H.N, Galanopoulos T., Neville-Golden J., Kiritsy C.P., Lynch S.E. (1991) Injury induces *in vivo* expression of platelet derived growth factor and platelet-derived growth factor receptor mRNAs in skin epithelial cells and platelet-derived growth factor mRNA in connective tissue fibroblasts. Proc. Natl. Acad. Sci. USA 88: 565-569.

Armelin H.A. (1973) Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc. Natl. Acad. Sci. USA 70: 2702-2706.

Assoian R.K., Fleurdelys B.E., Stevenson H.C., Miller P.J., Madtes D.K., Raines E.W., Ross R., Sporn M.B. (1987) Expression and secretion of type β transforming growth factor by activated human macrophages. Proc. Natl. Acad. Sci. USA. 84: 6020-6024.

Assoian R.K., Grotendorst G.R, Miller D.M., Sporn M.B. (1984) Cellular transformation by coordinated action of three peptide growth factors from human platelets. Nature. 309: 804-6.

Assoian R.K., & Sporn M.B. (1986) Type beta transforming growth factor in human platelets: Release during platelet degranulation and action on vascular smooth muscle cells. J. Cell Biol. 102: 1217-1223.

Babu M., Diegelmann R., Oliver N. (1992) Keloid fibroblasts exhibit an altered response to transforming growth factor β. J. Invest. Dermatol. 99: 650-655.

Bailey A..J., Sims T.J., Le Lous M., Bazin S. (1975b) Collagen polymorphism in experimental granulation tissue. Biochem. Biophys. Res. Commun. 66: 1160-1165.

Baird A, Mormede P, Bohlem P. (1985) Immunoreactive fibroblast growth factor in cells of peritoneal exudate suggests its identity in macrophage-derived growth factor. Biochem. Biophys Res. Commun. 126: 358-364.

Banda M. J. & Werb Z. (1981) Mouse macrophage elastase. Purification and characterisation of a metalloproteinase. Biochem. J. 193: 589-605.

Banda M.J., Knighton D.R., Hunt T.K., Werb Z. (1982) Isolation of a non-mitogenic angiogenesis factor from wound fluid. Proc. Natl. Acad. Sci. USA 79: 7773-7777.

Bar-Shavit R., Kahn A., Fenton J.W., Wilner G.D. (1983) Chemotactic response of monocytes to thrombin. J. Cell Biol. 96: 282-285.

Bassols A., & Massague J. (1988) Transforming growth factorβ regulates the expression and structure of the extracellular matrix chordrotin/dermatan sulfate proteoglycans. J. Biol. Chem. 263: 3039-45.

Bauer E.A., Cooper T.W., Huang J.S., Altman J., Deuel T.F. (1985) Stimulation of human *in vitro* human skin collagenase expression by platelet derived growth factor. Proc. Natl. Acad. Sci. USA 82: 4132-4136.

Beck L.S., Chen T.L., Mikalauski P., Ammann A.J. (1990) Recombinant humanTGFβ1 (rhTGFβ1) enhances healing and strength of granulation skin wounds. Growth factors. 3: 267-275.

Beck L.S., Deguzman L, Lee W.P., Xu Y., McFatridge L.A., Amento E.P. (1991) TGFβ1 accelerates wound healing: Reversal of steroid impaired healing in rats and rabbits. Growth Factors. 5: 295-304.

Bennett N.T. & Schultz G.S. (1993) Growth factors and wound healing: Part II: Role in normal and chronic wound healing. Am. J. Surg. 166: 74-81.

Bernstein E.F., Harisiadis L., Salomon G., Norton J., Sollberg S., Uitto J., Glatstein E., Glass J., Talbolt T., Russo A.., Mitchell J.B. (1991) TGFβ improves healing of radiation impaired wounds. J. Invest. Dermatol. 97: 430-434.

Bertone A.L. (1988) Control of granulation tissue on equine limb wounds: A clinical update. Vet Reports 1: 6.

Bertone A.L. (1989a) Principles of wound healing. Vet. Clin. North Amer. Equine Practice. 5: 3 449-463.

Bertone A.L. (1989b) Management of exuberant granulation tissue. 5: 3 551-562.

Bertone A.L., Sullins K.E., Stashak T.S. (1985) Effect of wound location and the use of topical collagen gels on exuberant granulation tissue formation and wound healing in the horse and pony. Am. J. Vet. Res. 46: 1438-1444.

Bevilacqua M.P., Amrami D., Mosesson M.W., Bianco C. (1981) Receptors for cold-insoluble globulin (plasma fibronectin) on human monocytes. J. Exp. Med. 153:42-60.

Birk D.E., Zycband E.I., Winklemann D.A., Trelstad R.L. (1989) Collagen fibrillogenesis in situ: Fibril segments are intermediates in assembly. Proc. Natl. Acad. Sci. USA 86: 4549-4553.

Birkedal-Hansen H. and Taylor R.E. (1982) Detergent-activation of latent collagenase and resolution of its component molecules. Biochem. Biophys. Res. Comm. 107: 1173-1178.

Blackford J., Shires M., Goble D., Geiser D., Held J.P. (1986) The use of N-butyl cyanoacrylate in the treatment of open leg wounds in the horse. In Prac. Am. Assoc. Equine Pract. 32: 329-356.

Blatti S.P., Foster D.N., Ranganathan G., Moses H.L., Getz M.J. (1988) Induction of fibronectin gene transcription and mRNA is a primary response to growth factor stimulation of AKR-2B cells. Proc. Natl. Acad. Sci. USA 85: 1119-23.

Blay J. & Brown K.D. (1985) Epidermal growth factor promotes the chemotactic migration of cultured rat intestinal epithelial cells. J. Cell Physiol. 24: 107-12.

Boone T.C., Johnson M.J., DeClerck. Y.A., Langley K.E. (1990) cDNA cloning and expression of a metalloproteinase inhibitor related to tissue inhibitor of metalloproteinases.. Proc. Natl. Acad. Sci. USA 87: 2800-2804.

Bordin S., Page R.C., Narayanan A.S. (1984) Heterogenicity of normal human diploid fibroblasts: isolation and characterisation of one phenotype. Science 223: 171-173.

Britton J.W. (1970) Wound management in horses. J.A.V.M.A. 157: 1585-1589.

Brown E.F. (1986) The role of extracellular matrix proteins in the control of phagocytosis. J. Leuk. Biol. 39: 579-591.

Browse N.L., Burnard K.G. (1982) The cause of venous ulceration. Lancet ii: 243-245.

Burnand K.G., Clemenson G., Whimster I., Gaunt J., Browse N.L. (1982) The effect of sustained venous hypertension on the skin capillaries of the canine hind limb. Br. J. Surg. 69: 41-44.

Busiek D.F., Ross F.P., McDonnell S., Murphy G., Matrisian L.M., Welgus H.G. (1992) The matrix metalloproteinase Matrilysin (Pump) is expressed in developing human mononuclear phagocytes. J. Biol. Chem. 267: 9087-9092.

CarricoT.J., Mehrhof A.I. & Cohen I.K. (1984) Biology of wound healing. Surg. Clin.N. Amer. 64: 721-733.

Cawston T.E., Murphy G., Mercer E., Galloway B.L., Hazleman B.L., Reynolds J. J. (1993) The interaction of purified rabbit bone collagenase with purified bone metalloproteinase inhibitor. Biochem. J. 211: 313-318.

Chua C.C., Geiman D.E., Keller G.H. Ladda R.L. (1985) Induction of collagenase secretion in human fibroblast cultures by growth promoting factors. J. Biol. Chem. 260: 5213-6.

Chvapil M., & Koopman C.F. (1984) Scar formation physiology and pathology states. Otolaryngol. Clin. North Am. 17: 265-272.

Chvapil M., Pfister T., Escalada S., Ludwig J., Peacock E.E.Jnr. (1979) Dynamics of the healing of skin wounds in the horse as compared to the rat. Exp. Mol. Pathol. 30: 349-359.

Clark R.A.F. (1983) Fibronectin in the skin. J. Invest. Dermatol. 81: 475-479.

Clark R.A.F. (1988) Potential roles of fibronectin in cutaneous wound repair. Arch Dermatol. 124: 201-206.

Clark R.A.F. (1990) Fibronectin matrix deposition and fibronectin receptor expression in healing and normal skin. J. Invest. Dermatol. 94: 128s-134s.

Clark R.A.F. (1993) Biology of dermal wound repair. Derm. Clin. 11: 647-666.

Clark R.A.F., Folkvord J.M., Neilson L.D. (1986) Either exogenous or endogenous fibronectin can promote adherence of human endothelial cells. J. Cell. Sci. 82: 263-280.

Clark R.A.F., Folkvard J.M., Wertz R.L. (1985) Fibronectin as well as other extracellular matrix proteins mediates human keratinocyte adherence. J. Invest. Dermatol. 84: 378-383.

Clark R.A.F., Lanigan J.M., Dellapella P., Manseau E., Dvorak H., Colvin R.B. (1982a) Fibronectin and fibrin provide a provisional matrix for epidermal cell migration during wound re-epithelisation. J. Invest. Dermatol. 79: 264-269.

Clark R.A.F., Stone R.D., Leung D.Y.K., Silver I.A., Hohn D., Hunt T.K. (1976) Role of macrophages in wound healing. Surg. Forum 27:16-18.

Clark R.A.F., Quinn J.H., Winn H.J., Lanigan J.M., Dellapella P., Colvin R.B. (1982b) Fibronectin is produced in blood vessels in response to injury. J. Exp. Med. 156: 646-651.

Claudy A.L., Mirshahi M., Soria C., Soria J. (1991) Detection of degraded fibrin and tumor necrosis factor-alpha in venous ulcers. J. Am. Acad. Dermatol. 25: 623-627.

Coffey R.J., Sipes N.J., Bascom C.C., Graves-Deal R., Pennington C.Y., Weissman B.E., Moses H.L. (1988) Growth modulation of mouse keratinocytes by transforming growth factors. Cancer Res. 48: 1596-602.

Cohen S. (1962) Isolation of a mouse submaxillary gland protein accelerating incisor eruption and eyelid opening in the newborn animal. J. Biol. Chem. 237: 1555-1562.

Cohen S. (1986) Epidermal growth factor. Biosci. Rep. 6: 1017-1028.

Cohen S. (1987) Epidermal growth factor. In vitro Cell Dev. Biol. 23: 239-50.

Cohen S., Carpenter G., Lembach K. J. (1975) Interaction of epidermal growth factor with cultured fibroblasts. Adv. Metab. Disord. 8: 265-284.

Collier I.E., Wilhelm S.M., Eisen A.Z., Marmer B.L., Grant G.A., Seltzer J.L., Kronberger A., He C., Bauer E.A., Goldberg G.I. (1988) Hras oncogene transformed human bronchial epithelial cells (TBE-1) secrete a single metalloproteinase capable of degrading basement membrane collagen. J. Biol. Chem. 263: 6579-6587.

Colvin R.B., Lanigan J.M. & Clarke R.A.F., Ebert T.H., Verderber E., Hammond M.E. (1979) Macrophage fibronectin (cold insoluble globulin. LETS- protein) Fed. Proc. 38:1408.

Compton C.C., Gill J.M., Bradford D.A., Regauer S., Gallico G.G., O'Connor N.E.. (1989) Skin regenerated from cultured epithelial autographs on full-thickness burn wounds from 6 days to 5 years after grafting: a light, electron microscope and immunohistochemical study. Lab Invest 60: 600-612.

Cordon-Cardo C., Vlodavsky I., Haimovitz-Friedman A., Hicklin D., Fuks Z. (1990) Expression of basic fibroblast growth factor in normal human tissue. Lab. Invest. 63: 832-840.

Couchman J.R., Austria M.R., Woods A.. (1990) Fibronectin-cell interactions. J. Invest. Dermatol. 1990; 94: 7s-14s.

Cox D.A., Kunz S., Vaxelaire J., Cerletti N., McMaster G.K., Burk R.R. (1993) Comparison of the effects of natural and recombinant transforming growth factorβ2 on wound healing responses in aged animals. Advances in Physiology of wound healing. (II): 135-139.

Curtsinger L.J., Pietsch J.D., Brown G.L., von-Fraunhofer A., Ackerman D., Polk H.C., Schultz G.S. (1989) Reversal of adriaycin impaired wound healing by transforming growth factor-beta. Surg. Gynecol. Obstet. 168: 517-522.

Davidson J. (1992) Wound repair. In: Inflammation. Basic principles and clinical correlates (ed. J.I. Gallin, I.M. Goldstein and R. Synderman) pp 809-819. Raven Press Ltd. New York.

Derynck R. (1988) Transforming growth factor α. Cell 54: 593-595.

Derynck R., Jarrett J.A., Chen E.Y., Eaton D.H., Bell J.R., Assoian R.K., Roberts A.B., Sporn M.B., Goeddel D.V. (1985) Human transforming growth factor-β complementary DNA sequence and expression in normal and transformed cells. Nature. 316: 701-705.

Deuel T.F. (1987) Polypeptide growth factors: Roles in normal and abnormal cell growth. Annu. Rev. Cell Biol. 3: 443-92.

Deuel T.F. & Huang J.S. (1984) Platelet derived growth factor: Structure, function and roles in normal and transformed cells. J. Clin. Invest. 74: 669-76.

Deuel T.F., Senior R.M.., Huang J.S., Griffin G.L. (1982) Chemotaxis of monocytes and neutrophils to PDGF. J. Clin. Invest. 69: 1046-1049. Diegelman R.F., Cohen I.K., Kaplan A.M. (1981) The role of macrophages in wound repair: a review. Plast. Reconstr. Surg. 68: 107-113.

Diegelmann R.F., Cohen I.K., Kaplan A.M. (1981) The role of macrophages in wound repair. A. Review. Plast. Reconstr. Surg. 68: 107-113.

Diegelmann R.F., Cohen I.K & McCoy B.J. (1979) Growth kinetics and collagen synthesis of normal skin, normal scar and keloid fibroblasts *in vitro*. J. Cell Physiol. 98: 341-346.

Docherty A.T.P., Lyons A.., Smith B.J., Wright E.M., Stephens P.E., Harris T.J.R., Murphy G., Reynolds J.J. (1985) Sequence of human tissue inhibitor of metalloproteinases and its identity to erythroid potentiating activity. Nature. 318: 66-69.

Docherty A.J.P. & Murphy G. (1990) The tissue metalloproteinase family and the inhibitor TIMP. A study using cDNAs and recombinant proteins. Ann. Rheum. Dis. 49: 469-479.

Docherty A.J.P., O'Connell J., Crabbe T., Angal S., Murphy G. (1992) The matrix metalloproteinases and their natural inhibitors: prospects for treating degenerative tissue disease. Trends in Biotech. 10: 200-207.

Doherty D.E., Haslet C., Tonnesen M.G., Henson P.M. (1987) Human monocyte adherence: A primary effect of chemotactic factors on the monocyte to stimulate adherence to human endothelium. J. Immunol. 138: 1762-1771.

Dvorak H.F. (1986) Tumous: Wounds that do not heal. New. Eng. J. Med. 315: 1650-1659.

Dyson M., Young S., Pendle C.L., Webster D.F., Lang S.M. (1988) Comparison of the effects of moist and dry conditions on dermal repair. J. Invest. Dermatol. 91: 434-439.

Edwards D.R., Murphy G., Reynolds J.J., Whitham S.E., Docherty A.J.P., Angel P., Heath J.K. (1987) Transforming growth factor β modulates the expression of collagenase and metalloproteinase inhibitor. EMBO J. 6: 1899-1904.

Epstein E.H. (1974) [αI(III)$_3$ human skin collagen: Release of pepsin digestion and preponderance in fetal life. J. Biol. chem. 249: 3225-3231.

Falanga V. (1993) Chronic wounds: Pathophysiologic and experimental considerations. J. Invest. Dermatol. 100: 721-725.

Fleischmajer R., Dessau W., Timpl R., Krieg T., Luderschmidt C., Wiestner M. (1980) Immunoflorescence analysis of collagen and fibronectin and basement membrane protein in sclerodermal skin. J. Invest. Dermatol. 75: 270-274.

Fleischmajer R., Perlish J.S., Krieg T., Timpl R. (1981) Variability in collagen and fibronectin synthesis by scleroderma fibroblasts in primary culture. J. Invest. Dermatol. 76:400-403.

Folkman J. & Klagbrun M. (1987) Angiogenic factors. Science 235: 442-7.

Folkman J., Klagsbrun M., Sasse J., Wadzinski M., Ingber D., Vlodavsky I. (1988) a heparin-binding Angiogenic protein, basic fibroblast growth factor, is stored within basement membrane. Am. J. Pathol. 130: 393-400.

Frankland A.L., Morris P.D.G., Spreull J.S.A. (1976) Free autologous skin transplantation in the horse. Vet. Rec. 98:105-110.

Fretz P.B., Martin G.S., Jacobs K.A., McIlwraith C.W. (1983) Treatment of exuberant granulation tissue in the horse: Evaluation of four methods. Vet. Surg. 12: 137-140.

Fukamizu H., & Grinnell F. (1990) Spatial organisation of extracellular matrix and fibroblast activity. Effects of serum TGFβ and fibronectin. Exp. Cell Res. 190: 276-83.

Gabbiani G. (1979) The role of contractile proteins in wound healing and fibrocontractive diseases. Meth.Achiev. Exp. Pathol. 9: 187-206.

Gabbiani G., Chapponinier C. and Huttner I. (1978) Cytoplasmic filaments and gap junctions in epithelial cells and myofibroblasts during wound healing. J. Cell. Biol. 76: 561-568.

Gabbiani G., Hirschel B.J., Ryan G.B., Statkov P.R., Majno G. (1972) Granulation tissue as a contractile organ. a study of structure and function. J. Exp. Med. 135: 719-734.

Gauss-Muller V., Kleinmann H.K., Martin G.R., Schiffman E. (1980) Role of attachment factors and attractants in fibroblast chemotaxis. J. Lab. Clin. Med. 96: 1071-1080.

Gay S., Viljanto J., Raeallio J., Penttinen R. (1978) Collagen types in early phases of wound healing in children. Acta. Chir. Scand. 144: 205-211.

Genetzky R.M., Biwer R.D., Myers R.K. (1983) Equine sarcoids. Causes, diagnosis and treatment. Compend. Cont. Educ. Pract. Vet. 5: S416-S420.

Gill G.N., Bertics P.J., Santon J.B. (1987) Epidermal growth factor and its receptor. Mol. Cell. Endocrinol. 51: 169-86.

Goldberg G.I., Strongin A., Collier I.E., Genrich L.T., and Marmer B.L. (1992) Interaction of 92kDa type IV collagenase with tissue inhibitor of metalloproteinases prevents dimerization, complex formation with interstitial collagenase, and activation of the proenzyme with stromelysin. J. Biol Chem. 267: 4583-4591.

Gonzalez A.M., Buscaglia M., Ong M., Baird A.. (1990) Distribution of basic fibroblast growth factor in 18-day rat fetus: Localisation in the basement membrane of diverse tissues. J. Cell Biol. 110: 753-765.

Goodson W.H. (1987) Application of expanded (ePTFE) tubes to study human wound healing. J. Biomaterials Applications. 2: 101-117.

Goodson W.H. & Hunt T.K. (1984) Wound healing in controlled diabetic mouse. Surg. Forum. 35:614-616.

Gorman N.T. (1985) Equine sarcoid. Time for optimism. Equine Vet. J. 17: 412-414.

Gospodarowicz D. (1974) Localisation of fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature. 249: 123-7.

Gospodarowicz D., Ferrara N., Schweigerer L., Neufeld G. (1987) Structural characterisation and biological functions of fibroblast growth factor. Endocrine reviews 95: 114.

Grant M.E. & Prockop D.J. (1972) The biosynthesis of collagen. N. Eng. J. Med. 286: 242-249.

Green H. & Goldberg B. (1964) Collagen and cellular protein synthesis by an established mammalian fibroblast line. Nature 204: 347-349.

Greenberg G.B. & Hunt T.K. (1978) The proliferative response *in vitro* of vascular endothelical cells and smooth muscle cells exposed to wound fluid and macrophages. J. Cell. Physiol. 97: 353-360.

Griffiths C.E.M., Baker J.N.W.N., Kunkel S., Nickoloff B.J. (1991) Modulation of leukocyte adhesion molecules, a T-cell chemotoxin (IL-8) and a regulatory cytokine (TNFα) in allergic contact dermatitis. Br. J. Dermatol. 124: 519-526.

Grinnell F., Billingham R.E., Burgess L. (1981) Distribution of fibronectin in epithelisation and wound healing. J. Invest Dermatol. 76: 181-189.

Grinnell F. & Feld M.K. (1979) Initial adhesion of human fibroblasts in serum-free medium: Possible role of secreted fibronectin. Cell 17: 117-129.

Grinnell F., Feld M. & Minster D. (1980) Fibroblast adhesion to fibrinogen and fibrin substrata: Requirement for cold-insoluble globulin (plasma fibronectin). Cell 19: 517-525.

Grinnell F., Ho C.H., & Wysocki A. (1992) Degradation of fibronectin and vitronectin in chronic wound fluid: Analysis by cell blotting, immunoblotting and cell adhesion assays. J. Invest. Dermatol. 98: 410-416.

Grinnnell F. & Lamke C.R. (1984) Reorganisation of hydrated collagen lattices by human skin fibroblasts. J. Cell Sci. 66: 51-63.

Grotendorst G.R., Cheng T., Seppa H.E.J., Kleinman H.K., Martin G.R. (1982) Platelet derived growth factor is a chemoattractant for vascular smooth muscle cells. J. Cell. Physiol. 113: 261-266.

Grotendorst G.R., Seppa H.E.J., Kleinman H.K., Martin G.R. (1981) Attachment of smooth muscle cells to collagens and their migration toward platelet-derived growth factor. Proc. Natl. Acad. Sci. USA. 78: 3669-3672.

Grotendorst G.R., Soma Y., Takehara K., Charette M. (1989) Epidermal growth factor and Transforming growth factor α are potent chemoattractants for endothelial cells and EGF-like peptides are present at sites of tissue regeneration. J. Cell Physiol. 139: 617-23.

Grotendorst G.R., Pencev D., Martin G.R., Sodek J. (1984) Molecular mediators of tissue repair. (In: Hunt, Heppenstall, Pine etc eds) Soft and hard tissue repair: Biological and clinical aspects. New York: Praeger pp 20-41.

Groves R.W., Allen M.H., Barker J.N.W.N., Haskard D.O., MacDonald D.M. (1991) Endothelial leukocytes adhesion mol-1 (ELAM 1) expression in cutaneous inflammation. Br. J. Dermatol. 124: 117-123.

Hammacher A., Hellman U., Johnsson A., Oestman A., Gunnarsson K., Westermark B., Wasteson A., Heldin C.H. (1988) A major part of PDGF purified from human platelets is a heterodimer of one A & B chain. J. Biol. Chem. 263: 16493-8.

Harper R.A. & Grove G. (1979) Human skin fibroblasts derived from papillary and reticular dermis: Differences in growth potential *in vitro*. Science 204: 526-527.

Harris E.D. & Krane S.M. (1971) Effects of colchicine on collagenase in cultures of rheumatoid synovium. Arthritis Rheum. 14: 669-684.

Hasty K.A., Hibbs M.S., Kang A.H., Mainardi C.L. (1986) Secreted forms of human neutrophil collagenase. J. Biol. Chem. 261: 5645-5650.

Hasty K.A., Pourmotabbed T.F., Goldberg G.I. Thompson J.P., Spinella D.G., Stevens R.M., Mainardi C.L. (1990) Human neutrophil collagenase. A distinct gene product with homology to their matrix metalloproteinases. J. Biol. Chem. 265: 11421-11424.

Haukipuro K., Melkko J., Risteli L., Kairaluoma M. I., Risteli J. (1991) Synthesis of type I collagen in healing wounds in humans. Ann. Surg. 213: 75-80.

Haydock D.A. & Hill G.L. (1987) Improved wound healing responses in surgical patients receiving intravenous nutrition. Br. J. Surg. 74: 320 -323.

Hebda P.A., Klingbeil C.K., Abraham J.A., Fiddes J.C. (1990) Basic fibroblast growth factor stimulation of wound healing in pigs J. Invest. Dermatol. 95: 626-31

Heldin C.H., Backstrom G., Ostman A., Hammacher, A., Ronnstrand L., Rubin K., Nister M., Westermark B. (1988) Binding of different dimeric forms of PDGF to human fibroblasts: Evidence for two separate receptor forms. EMBO J. 7: 1387-93.

Heldin P., Laurent T.C., Heldin C.H. (1989) Effect of growth factors on hyaluronan synthesis in cultured human fibroblasts. Biochem. J. 258: 919-22.

Henkel W. & Glanville R.W. (1982) Covalent crosslinking between molecules of type I and type III collagen. Eur. J. Biochem. 122: 205-213.

Herrick S.E., Sloan P., McGurk M., Feak L., McCollum C.N., Ferguson M.W.J. (1992) Sequential changes in the histological pattern and extracellular matrix deposition during the healing of chronic venous ulcers. Am. J. Pathol. 141: 1085-1095.

Hibbs M.S., Hasty K.A., Seyer J.M. Kang A.H. Maincardi C.L. (1985) Biochemical immunological characterisation of secreted forms of human neutrophil gelatinase. J. Biol. Chem. 260: 2493-2500.

Highly H., Ksander G., Gerhart C., Kirsner R., Falanga V. (1992) Immunochemical analysis of growth factors and growth factor response cells in chronic venous stasis ulcers. Wounds 4: 33-34.

Hoffman K.D., Kainer R.A., Shideler R.K. (1983) Radio frequency current-induced hyperthermia for the treatment of equine sarcoid. Equine Practice. 5: 24-31.

Hogle R., Kingrey B.W., Jensen E.C. (1959) Skin grafting in the horse. J.A.V.M.A. 165-170.

Howard E.W. & Banda M.J. (1991) Binding of tissue inhibitor of MMP2 to two distinct sites on human 72kDa gelatinase: identification of a stabilisation site. J. Biol. Chem. 266: 17972-17977.

Hsieh P.& Chen L.B. (1983) Behaviour of cells seeded on isolated fibronectin matrices. J. Cell Biol. 96: 1208-1217.

Hunt T.K. (1980) Wound healing and wound infection: Theory and surgical practice. New York, Appleton Century-Crofts.

Hunt T.K. (1991) Vascular factors govern healing in chronic wounds. In Clinical and experimental approaches to dermal and epithelial repair: Normal and chronic wounds (ed. A.Barbul, M.Caldwell, W. Eaglestein, T.K. Hunt, D. Marshall, E. Pines, G. Skover) John Wiley & Sons: New York, pp 1-17.

Hunt T.M., Connolly W.B., Aronson S.B., Goldstein P. (1978) Anaerobic metabolism and wound healing: A hypothesis for the initiation and cessation of collagen synthesis in wounds. Am. J. Surg. 135: 328-332.

Hunt T.K., Knighton D.R., Thakral K.K., Andrews W., Michaeli D. (1984) Cellular control of repair In: Soft and hard tissue repair (T.K. Hunt, Heppenstall, Pines, Rovee eds) pp 3-19 Praeger New York.

Hunt T.K. & Van Winkle W. Jnr. (1979) Normal repair: In fundamentals of wound management. (Eds. T.K. Hunt & J.E. Dunphy) pp 2-67. Appleton-Century Crofts: New York.

Hynes R.O. (1973) Alteration of cell-surface protein by viral transformation and by proteolysis. Proc. Natl. Acad. Sci. 70: 3170-3174.

Hynes R.O. (1990) Fibronectins. Springer-Verlag: New York.

Ignotz R.A., Endo T., & Massague J. (1987) Regulation of fibronectin and type I collagen mRNA levels by TGFβ. J. Biol. Chem. 262: 6443-6.

Ignotz R.A. & Massague J. (1986) Transforming growth factor-β stimulates the expression of fibronectin and collagen and their incorporation into the extacellular matrix. J. Biol. Chem. 261: 4337-4345.

Ishai-Michaeli R., Eldor A., Vlodasky I. (1990) Heparanase activity expressed by platelets, neutrophils, and lymphoma cells releases active fibroblast growth factor from extracellular matrix. Cell Reg. 1: 833-842.

Jackson C. (1936) The incidence and pathology of tumours of domestic animals in South Africa. A study of the Onderstepoort collection of neoplasms with special reference to their histopathology. Onderstepoort. J.Vet. Res. 6: 1-460.

Jacobs K.A., Leach D.H., Fretz P.B., Townsend H.G.G. (1984) Comparative aspects of the healing of excisional wounds on the leg and body of horses. Vet. Surg. 13: 83.

Jaffe E.A., Leung L.L.K., Nachman R.L., Levin R.I., Mosher D.F. (1982) Thrombospondin is the endogenous lectin of human platelets. Nature 295: 246-248.

Jaffe E.A. & Mosher D.F. (1978) Synthesis of fibronectin by cultured human endothelial cells. J. Exp. Med. 147: 1779-1791.

Jalkanen M., Haapanen T., Lyytikainen A.M., Larjava H. (1983) Wound fluids mediate granulation tissue growth phases. Cell Biol. Int. Rep. 7: 745-753.

Jensen J.A., Goodson W.H., Vasconez L.O., Hunt T.K. (1986) Wound healing in anaemia. a case report. West. J. Med. 144: 465-467.

Johnsson A., Heldin C.H., Westermark B., Wasteson A.. (1982) Platelet derived growth factor: Identification of constituent polypeptide characteristics. Biochem. Biophys. Res. Commun. 104: 66-74.

Juva K. & Prockop D.J. (1966) Modified procedure for the assay of H^3- or C14- labelled hydroxyproline. Anal. Biochem. 15: 77-83.

Kane C.J.M., Hebda P.A., Mansbridge J.N., Hanawalt P.C. (1991) Direct evidence for spatial and temporal regulation of TGFβ1 expression during cutaneous wound healing. J. Cell Physiol. 148: 157-173.

Kanzler M.H., Gorsulowsky D.C., Swanson N.A.. (1986) Basic mechanisms in the healing cutaneous wound. J.Dermatol. Surg. Incol. 12: 1156-1164.

Kasselberg A.G., Orth D.N., Gray M.E., Stahlman M.T. (1985) Immunochemical localisation of human EGF/urgastrone in several human tissues. J. Histochem. Cytochem. 33: 315-322.

Katz M.H., Alvarez A.F., Kirsner R.S., Eaglestein W.H., Falanga V. (1991) Human wound fluid from acute wounds stimulates fibroblast endothelial cell growth. J. Am. Acad. Dermatol. 25: 1054-1058.

Kingsnorth A.N. and Slavin J. (1991) Peptide growth factors and wound healing. Br. J. Surg. 78: 1286-1290.

Kischer C.W., Shetlar M.R., Chvapil M. (1982) Hypertrophic scars and keloids: A review and new concept concerning their origin. Scan. Electron. Microsc. 4: 1699-1713.

Kjeldsen L., Sengelov H., Lollike K., Neilsen M.H., Borregaard N. (1994) Isolation and characterisation of gelatinase granules from human neutrophils. Blood 83: 1640-1649.

Klinbeil E.A. , Cesar T., Fiddes J. (1991) Basic fibroblast growth factor accelerates tissue repair in models of impaired healing. In:(Barbul, Caldwell, Eaglestein eds) Clinical and experimental approaches to dermal and epidermal repair: Normal and chronic wounds. New York. John Wiley & Sons. pp 443-58.

Knighton D.R., Cirese K., Figel V.D., Schumerth S., Butler E., Cerra F. (1990) Stimulation of repair in chronic, non-healing cutaneous ulcers using platelet derived wound healing formula. Surg. Gynaecol. Obstet. 170: 56-60.

Knottenbelt D.C., Edwards S. & Daniel E. (1995) Diagnosis and treatment of the equine sarcoid. In Practice 17:(3) 123-129.

Knottenbelt D.C. & Pascoe R.R. (1994) Color Atlas of Diseases and Disorders of the Horse. (London, Mosby-Wolfe).

Knottenbelt D.C. & Walker J.A. (1994) Topical treatment of the equine sarcoid. Equine vet. Edu. 6: 72-75.

Koch A.E., Burrows J.C., Haines G.K., Carlos T.M., Harlan J.M., Leibovich S.J. (1991) Immunolocalisation of endothelial and leukocyte adhesion molecules in human rheumatoid and osteoarthritic synovial tissues. Lab. Invest. 64: 313-320.

Ksander G.A., Ogawa Y., Chu G.H., McMillin H., Rosenblatt J.S., McPherson J.M. (1990) Exogenous transforming growth factor-beta 2 enhances connective tissue formation and wound strength in guinea pigs dermal wounds healing by secondary intent. Ann. Surg. 211: 288-294.

Kubo M., Norris D.A., Howell S.E., Ryan S.R., Clarke R.A.F. (1984) Human keratinocytes synthesise, secrete and deposit fibronectin in the pericellular matrix. J. Invest. Dermatol. 82: 580-586.

Kurkinsen M., Vaheri A., Roberts P.J., Stenman S. (1980) Sequential appearance of fibronectin and collagen in experimental granulation tissue. Lab. Invest. 43: 47-51.

Laemmli, U.K. (1970) Cleavage of proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Lancaster W.D. (1981) Apparent lack of BPV DNA in virus induced equine and bovine tumour cells and virus transformed mouse cells. Virology. 108: 251-255.

Lancaster W.D., Olsen C., Meinke W. (1977) Bovine papilloma virus, presence of virus specific DNA sequences in naturally occurring equine tumours. Proc. Natl. Acad. Sci. USA 74: 524-528.

Lancaster W.D., Theilen G.H., Olsen C. (1979) Hybridisation of BPV type 1 and type 2 DNA to DNA from virus induced hamster tumours and naturally occurring equine tumours. Intervirology. 11: 227-233.

Lane J.G. (1977) The treatment of equine sarcoids by cryosurgery. Equine Vet. J. 9: 127-133.

Langness U. & Udenfriend S. *(1973) Collagen proline hydroxylase activity and* anaerobic metabolism. In: Biology of the fibroblast (R. Kulonen & J. Pikkarainen eds) pp 373-378 Academic New York.

Lanir N., Ciano P.S., Van der Water L., McDonagh J., Dvorak A.M., Dvorak H.F. (1988) Macrophage migration in fibrin gel matrices: II. Effects of clotting factor XIII, fibronectin and glycosaminoglycan content on cell migraton. J. Immunol. 140: 2340-2349.

Lark M.W., Laterra J. & Culp L.A. (1985) Close and focal contact adhesions of fibroblasts to fibronectin-containing matrix. Fed. Proc. 44: 394-403.

Lawrence W.T., Sporn M.B., Gorschboth C., Norton J.A., Grotendorst G.R. (1986) The reversal of an Adriamycin induced healing impairment with chemoattractants and growth factors. Ann. Surg. 203: 142-147.

Leach R.D. (1984) Venous ulceration, fibrinogen and fibrinolysis. Ann. Rev. Coll. Surg. Eng. 66: 258-263.

Lee A..H., & Swain S.F. (1988) Granulation tissue: How to take advantage of it in the management of open wounds. Comp. Contin. Educ. Pract. Vet. 10:163.

Leitzel K., Cono C., Marks J.G. Jnr., Lipton A.. (1985) Growth factors and wound healing in the hamster. J. Dermatol. Surg. Oncol. 11: 615-622.

LeRoy E.C. (1974) Increased collagen synthesis by scleroderma skin fibroblasts *in vitro*. A positive defect in the regulation or activation of the scleroderma fibroblast. J. Clin. Invest. 54: 880-889.

Leu H.J. (1991) Morphology of chronic ulcers insufficiency - light and electron microscopic examinations. VASA 20: 330-342.

Levene C.I. & Bates C.J. (1976) The effect of hypoxia on collagen synthesis in cultured 3T6 fibroblasts and its relationship to the mode of action of ascorbate. Biochem. Biophys. Acta. 444: 446-452.

Levenson S.M., Geever E.F., Crowley L.V., Oates J.F., Berard C.W., Rosen H. (1965) The healing of rat skin wounds. Ann Surg. 161: 293-308.

Levine J.H., Moses H.L., Gold L.I., Nanney L.B. (1993) Spatial and temporal patterns of immunoreactive TGFβ1, β2 and β3 during excisional wound repair. Am. J. Pathol. 143: 368-380.

Liebovich S.J. (1984) Mesenchymal cell proliferation in wound repair: The role of macrophages In: Soft and hard tissue repair (T.K. Hunt, R.B. Heppstall, Pines, rovee eds) pp 329-351 Praeger New York.

Liebovich S.J. & Ross R. (1976) A macrophage dependant factor that stimulates the proliferation of fibroblasts *in vitro*. Am. J. Pathol. 84: 501-513.

Lim R.W. & Hauschka S.D. (1984) A rapid decrease in EGF binding capacity accompanies the terminal differentiation of mouse myofibroblasts *in vitro*. J. Cell Biol. 98: 739-347.

Lindsay W.A. (1988) Wound treatment in horses. What to know about second intension healing. Equine Pract. 10: 396.

Loef E.B., Proper J.A., Goustin A.S., Shipley G.D., DoCorleto P.E., Moses H.L. (1986) Induction of C-sis mRNA and activity to platelet derived growth factor-like activity by transforming growth factor type-β: A proposed model for indirect mitogenesis involving autocrine activity. Proc. Natl. Acad. Sci. USA 83: 2453-2457.

Lowry O.H., Roseborough N.J., Farr A.L., Randall R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275.

Lyons R.M. & Moses H.L. (1990) Transforming growth factors and the regulation of cell proliferation. Eur. J. Biochem. 187: 467-73.

Macarak E.J. & Howard P.S. (1983) Adhesion of endothelial cells to extracellular matrix proteins. J. Cell Physiol. 116: 76-86.

Macarak E.J., Kirby E., Kirk T., Kefalides N.A. (1978) Synthesis of cold-insoluble globulin by cultured calf endothelial cells. Proc. Natl. Acad. Sci. USA. 75: 2621-2625.

Mackie E.J., Halfter W. & Liverani D. (1988) Induction of tenascin in healing wounds. J. Cell. Biol. 107: 2757-2767.

Majno G., Gabbiani G., Hirschel B.J., Ryan G.B., Statkov P.R. (1971) Contraction of granulation tissue *in vitro*: Similarity to smooth muscle. Science 173: 548-550.

Marti E., Lazary S., Antczak D.F., Gerber H. (1993) Report of the first international workshop on the equine sarcoid. Equine Vet. J. 25: 397-407.

Martin B.M., Gimbrone M.A. Jnr., Unanue,E.R., Cotran R.S. (1981) Stimulation of non-lymphoid mesenchymal cell proliferation by a macrophage-derived growth factor. J Immunol. 126: 1510-1515.

Martin G.R., Timpl R., Muller P.K., Kuhen K. (1985) The genetically distinct collagens. Trans. Int. Biol. Soc. 115: 285-287.

Martinet Y., Bitterman P.B., Mornex J.F., Grotendorst G.R., Martin G.R., Crystal R.G. (1986) Activated human monocytes express the C-SIS protooncogene to release a mediator showing PDGF-like activity. Nature. 319: 158-60.

Massague J. (1990) The transforming growth factor β family. Annu. Rev. Cell Biol. 6: 597-641.

Massague J., Cheifetz S., Ignotz R.A., Boyd F.T. (1987) Multiple type β transforming growth factors and their receptors. J. Cell Physiol. (Suppli) 5: 43-7.

Matsubara M., Girard M.T., Kublin C.L., Cintron C., Fini M.E. (1991) Differential roles for two gelatinolytic enzymes of the MMP family in the remodelling cornea. Dev. Biol. 147: 425-439.

McAuslan B.R., Bender V., Reilly W., Moss B.A. (1985) New functions of epidermal growth factor stimulation of capillary endothelial cell migration and matrix dependant proliferation. Cell Biol. Int. Rep. 9: 175-82.

McCartney-Francis N., Mizel D., Wong H., Wahl L., Wahl S. (1988) Transforming growth factor β as an immunoregulatory molecule. FASEB J. 2: a 875.

Mc Donald J.A., Kelly D.G. & Broekelmann T.J. (1982) Role of fibronectin in collagen deposition:Fab' to the gelatin-binding domain of fibronectin inhibits both fibronectin and collagen organisation in fibroblast extracellular matrix. J. Cell Biol. 92: 485-492.

McGee G .S. Davidson J.M., Buckley A., Sommer A, Woodward S.C., Aqino A.M., Barbour R., Demetriou A..A. (1988) Recombinant basic fibroblast growth factor accelerates wound repair. J. Surg. Res. 45: 145-53.

McGrath M.H. (1990) Peptide growth factors and wound healing. Clin Plast. Surg. 17: 421-432.

Meagher D.M. & Adams O.R. (1971) Split-thickness autologous skin transplantation in the horse. J. Am. Vet. Med. Assoc. 159: 55-60.

Miller E.J. (1976) Biochemical characteristics and biological significance of genetically distinct collagens. Molec. Cell. Biochem. 13: 165-189.

Miyazono K., Ichijo H. & Heldin C. (1993) Transforming growth factor -β: Latent forms, binding proteins and receptors. Growth factors. 8: 11-22.

Moscatelli D. & Rifkin D.B. (1988) Membrane and matrix localisation of proteinases. A common theme in tumor cell invasion and angiogenesis. Biochem. Biophys. Acta. 948: 67-85.

Moses H.L., Tucker R.F., Leof E.B., Coffey R.J., Halper J., Shipley G.D. (1985) Type beta transforming growth factor is a growth stimulator and growth inhibitor. Cancer Cells (Old Spring Harbor) 3: 65-71.

Mosher D.F. (1975) Cross-linking of cold-soluble globulin by fibrin-stabilising factor. J. Biol. Chem. 250: 6614-6621.

Mosher D.F. (1989) Fibronecin. Academic Press. New York.

Mosher D.F. & Vaheri A. (1978) Thrombin stimulates the production and release of a major surface-assay glycoprotein in cultures of human fibroblasts. Exp. Cell. Res. 112: 323-334.

Murphy G., Crockett M.I., Stephens P.E., Smith B.J., Docherty A.J.P. (1987) Stromelysin is an activator of procollagenase. A study with natural and recombinant enzymes. Biochem. J. 248: 265-268.

Murphy G., Willenbrock F., Ward R.V., Cockett M.I., Eaton D., Doherty A.J.P. (1992) The C-terminal domain of 72kDa gelatinase is not required for catalysis but is essential for membrane activation and modulates interactions with tissue inhibitors of MMPs. Biochem. J. 283: 637-641.

Mustoe T.A., Pierce G.F., Morishima C., Deuel T.F. (1991) Growth factor induced acceleration of wound repair through direct and inductive activities in a rabbit dermal ulcer model. J. Clin. Invest. 87: 694-703.

Mustoe T.A., Pierce G.F., Thomasen A., Gramates P., Sporn M.B., Deuel T.F. (1987) Accelerated healing in incisional wounds in rats induced by TGFβ. Science 237: 1333-1336.

Nagase H., Okada Y., Suzuki K., Enghild J.J., Salvesen G. (1991) Substrate specificities and activation mechanisms of matrix metalloproteinses. Bioch. Soc. Trans. 19: 715-718.

Nakagawa S., Yoshida S., Hirao Y., Kasuga S., Fuwa T. (1985) Biological effects of biosynthetic human epidermal growth factor on the growth of mammalian cells *in vitro*. Differentiation. 29: 284-8

Nanney L.B., Magid M., Stoscheck C.M., King L.E. Jnr. (1984) Comparison of EGF binding and receptor distribution in normal human epidermis and epidermal appendages. J. Invest. Dermatol. 83: 385-393.

Neumann H.A.M & Van den Broek M.J.T.B. (1991) Increased collagen IV layer in the basal membrane area of the capillaries in severe chronic venous insufficiency. VASA 20: 26-29.

Newman S.L., Henson J.E., & Henson P.M. (1982) Phagocytosis of senescent neutrophils by human monocyte-derived macrophages and rabbit-inflammatory macrophages. J. Exp. Med. 156: 430-442.

Niinikowski J., Gothrup F. & Hunt T.K. (1991) The role of oxygen in wound repair. In: Wound healing (eds. H. Janssen, R.Rooman, J.I.S. Robertson) pp 165-177. Wrightson: Petersfield U.K.

Nishiyama T., Akutsu N., Horii I., Nakayama Y., Ozawa T., Hayashi T. (1991) Response to growth factors of human dermal fibroblasts in quiescent state owing to cell-matrix contact inhibition. Matrix 11: 71-75.

Nomura H., Sato H., Seiki M., Mai M., Okada Y. (1995) Expression of membrane-type matrix metalloproteinase in human gastric carcinomas. Cancer Res. 55: 595-598.

Norris D.A., Clark R.A.F., Swigart L.M., Huff J.C. Weston W.L., Howell S.E. (1982) Fibronectin fragments are chemotactic for peripheral blood monocytes. J. Immunol. 129: 1612-1618.

Norris P., Poston R.N., Thomas D.S., Thornhill M., Hawk J., Haskard D.O. (1991) The expression of endothelial leukocyte adhesion molecule-1(ELAM-1), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in experimental cutaneous inflammation: a comparison of ultraviolet B erythema and delayed hypersensitivity. J. Invest. Dermatol. 96: 763-770.

Oikarinen A.I., Kylmaniemi M., Autio-Harmainen H., Autio P., Salo T. (1993) Demonstration of 72 and 92 kDa forms of type IV collagenase in human skin: Variable expression in various blistering diseases, induced during re-epithelisation and decrease by topical glucocorticoids. J. Invest. Dermatol. 101: 205-210.

Oikarinen A.I., Zone J.J., Ahmed A.R., Kiistala, U., Uitto J. (1983) Demonstration of collagenase and elastase activities in the blister wound fluids from bullous skin diseases. Comparison between dermatitis herpetisformis and bullous pemphigold. J. Invest. Dermatol. 81: 261-266.

Oka Y. & Orth D.N. (1983) Human plasma EGF/urgastrone is associated with blood platelets. J. Clin. Invest.72: 249-259.

O'Keefe E.J., Chiu M.L., Payne R.E. Jnr. (1988) Stimulation of growth of keratinocytes by basic fibroblast growth factor. J. Invest. Dermatol. 90: 767-769.

O'Keefe E.J., Payne R.E. Jr., Russell N., Woodley D.T. (1985) Spreading and enhanced motility of human keratinocytes on fibronectin. J. Invest. Dermatol. 85: 125-130.

Olsen C. (1948) Equine sarcoid, a cutaneous neoplasm. Am. J. Vet Res. 9:333-341.

Olsen C. Jnr. & Cook R.H. (1951) Cutaneous sarcoma-like lesions of the horse caused by the agent bovine papilloma. Proc. Soc. Exp. Biol. Med. 77: 281-284.

Overall C.M., Wrana J.L. & Sodek J. (1989) Transforming growth factor beta regulation of collagenase, 72kDa- progelatinase, TIMP and PA1 expression in rat bone cell populations and human fibroblasts. Connect. Tiss. Res. 20: 289-94.

Pardes J.B., Tonneson M.G., Falanga V., Eaglestein W.H. & Clark R.A.F. (1990) Skin capillaries surrounding chronic ulcers demonstrate smooth muscle cell hyperplasia and increased laminin and type IV collagen. J. Invest. Dermatol. 94: 563.

Peacock E.E. (1973) Biology of wound repair. Life Sci.13: 1-9.

Peacock E.E., Madden J.W., Trier W.C. (1970) Biological basis for the treatment of keloids and hypertrophic scars. South Med. J. 63: 755-760.

Pesonen K., Viinikka L., Myllyla G., Kiuru J., Perheentupa J. (1991) Characterisation of material with EGF immunoreactivity in human serum and platelets. J. Clin. Endocrinol. Metab. 68: 486-491.

Peterkovsky B. (1972) The effects of ascorbic acid on collagen peptide synthesis and proline hydroxylation during the growth of cultured fibroblasts. Arch. Biochem. Biophys. 152: 318-328.

Peterkovsky B. & Diegelmann R. (1971) Use of a mixture of proteinase-free collagenases for the specific assay of radioactive collagen in the presence of other proteins. Biochemistry 10: 988-994.

Picker L.J., Kishimoto T.K., Smith C.W., Warnock R.A., Butcher E.C. (1991) ELAM-1 is an adhesive molecule for skin-homing T cells. Nature 349: 796-799.

Pierce G.F., Mustoe T.A., Lingelbach J., Masakowski V.R., Gramates P., Senior R.M., Deuel T.F. (1989) Transforming growth factor-β reverses the glucocorticoid-induced wound healing deficit in rats: Possible regulation in macrophages by PDGF. Proc. Natl. Acad. Sci. USA 86: 2229-2233.

Pierce G.F., Mustoe T.A., Lingelbach J., Masakowski V.R., Griffin G.L., Senior R.M., Deuel T.F. (1986) Platelet-derived growth factor and Transforming growth factor-β enhance tissue repair activation by unique mechanisms. J. Cell. Biol. 109: 429-440.

Pierce G.F., Tarpley J.E., YanagiharaD., Mustoe T.A., Fox G.M., Thomason A. (1992) Platelet derived growth factor (BB homodimer) Transforming growth factor β1 and basic fibroblast growth factor in dermal wound healing. Am J. Pathol.140: 1375-1388.

Pober J.S., Cotran R.S. (1991) What can be learned from the expression of endothelial adhesion molecules in tissues? Lab Invest. 64: 301-305.

Postlethwaite A.E.& Kang A.H. (1976) Collagen and collagen peptide-induced chemotaxis of human blood monocytes. J. Exp. Med. 143: 1299-1307.

Postlethwaite A.E., Keski-Oja J., Moses H.L., Kang A.H. (1987) Stimulation of chemotactic migration of fibroblasts by transforming growth factor β. J. Exp. Med. 165: 251-256.

Postlethwaite A.E., Synderman R., Kang A.H. (1976) The chemotactic attraction of human fibroblasts to lymphocyte derived factor. J. Exp. Med. 144: 1188-1203.

Quaglino D., Nanney L.B., Ditesheim J.A., Davidson J.M. (1991)Transforming growth factor-beta stimulates wound healing and modultes extracellular matrix gene expression in pig skin incisional wound model. J. Invest. Dermatol. 97: 34-42.

Quantin B., Murphy G., Breathnach R. (1989) Pump 1 cDNA codes for a protein with characteristics similar to those of classical collagenase family members. Biochemistry 28: 5327-5334.

Raghow R., Postlethwaite A.E., Keshi-Oja J., Moses H.L., Kang A..H. (1987) Transforming growth factor-β increases steady state levels of type I procollagen and fibronectin messenger RNA's in dermal fibroblasts. J. Clin. Invest. 79: 1285-1288.

Ragland W.L. & Spencer G.R. (1968) Attempts to relate bovine papilloma virus to the cause of equine sarcoid. Immunity to bovine papilloma virus. Am. J. Vet. Res. 29: 1363-1366.

Ragland W.L. & Spencer G.R. (1969) Attempts to relate bovine papilloma virus to the cause of equine sarcoid. Equidae inoculated intradermally with bovine papilloma virus. Am. J. Vet. Res. 30: 743-751.

Rasmussen L.H., Jensen L.T., Avnstorp C., Karlsmark T., Peters K., Horslev-Petersen K. (1992) Collagen types I and III propeptides as markers of healing in chronic leg ulcers. Ann. Surg. 216: 684-691.

Read A.E. (1987) What happened to Urogastrone? Q. J. Med. 62: 1-6.

Regan M.C., Kirk S.J., Wasserkrug B.A., Barbul A. (1991) The wound environment as a regulator of fibroblast phenotype. J. Surg. Res. 50: 442-448.

Reid S.W.J., Gettinby G., Fowler J.N., Ikin P. (1994) Epidemiological observations on sarcoids in a population of donkeys (equus asinus). Vet. Rec. 134: 207-211.

Rice G.E., Munro J.M., Corless C., Bevilacqua M.P. (1991) Vascular and non-vascular expression of ICAM-110: A target for mononuclear leucocyte adhesion in normal and inflamed human tissue. Am J. Pathol. 138: 385-393.

Rizzino A. (1988) Transforming growth factor-β: Multiple effects on cell differentiation and extracellular matrices. Dev. Biol.130: 411-422.

Roberts A.B., Anzano M.A., Wakefield L.M., Roche N.S., Stern D.F., Sporn M.B. (1985) Type β Transforming growth factor: A bifunctional regulator of cellular growth Proc. Natl. Acad. Sci. USA 82: 119-23.

Roberts A.B., Sporn M.B., Assoian R.K., Smith J.M., Roche N.S., Wakefield L.M., Heine U.I., Liotta L.A., Falanga V., Kehrl J.H., Fauci A.S. (1986) Transforming growth factorβ: Rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro*. Proc. Natl. Acad. Sci. USA 83: 4167-4171.

Robson M.C., Phillips L.G., Cooper D.M. (1993) Transforming growth factor-beta 2 accelerates healing of venous stasis ulcers in an open-label placebo-controlled clinical study. Wound Repair and Regeneration. 1: 91.

Rockwell W.B., Cohen I.K., Ehrlich H.P. (1989) Keloids and hypertrophic scars: A review. Plast. Reconstr. Surg. 84: 827-837.

Ross R. (1987) Platelet derived growth factor. Annu. Rev. Med. 38: 716-9.

Ross R. & Benditt E.P. (1961) Wound healing and collagen formation. Sequential changes in components of guinea pig skin wounds observed in the electron microscope. J. Biophys. Biochem. 11: 677-700.

Ross R., Glomset J., Kariya B., Harker L. (1974) A platelet-dependent serum factor that stimulates the proliferation of arterial smooth muscle cells in vitro. Proc. Natl. Acad. Sci.71: 1207-1210.

Ross R., Raines E.W. & Bowen-Pope D.F. (1986) The biology of platelet-derived growth factor. Cell 46: 155-169.

Ruoslahti E. & Vaheri A. (1974) Novel human serum protein from fibroblast plasma membranes. Nature. 248: 789-791.

Rutherford R.B. & Ross R. (1976) Platelet factors stimulate fibroblasts and smooth muscle cells quiescent in plasma serum to proliferate. J. Cell Biol. 69: 196-203.

Saarialho-Kere U.K., Chang E.S., Welgus H.G., Parks W.C. (1992) Distinct localisation of collagenase and tissue inhibitor of MMP expression in wound healing associated with ulcerative pyrogenic granuloma. J. Clin. Invest. 90: 1952-1957.

Sakata K., Hoshino K., Nakagawa H. (1989) Purification and characterisation of exudate gelatinases in chronic phase of carrageenin-induced inflammation in rats. J. Biochem. 105: 384-389.

Saksela O. & Rifkin D.B. (1990) Release of bFGF heparan sulphate complexes from endothelial cells by plasminogen activator mediated proteolytic activity. J. Cell. Biol. 105: 957-963.

Salo T., Lyons J.G., Rahemtula F., Birkedal-Hansen H., Larjava H. (1991) Transforming growth factor β upregulates type IV collagenase expression in cultured human keratinocytes. J. Biol. Chem. 266: 11436-11441.

Salo T., Makela M., Kylmaniemi M., Autio-Harmainen H., Larjava H. (1994) Expression of MMP 2 and MMP9 during early human wound healing. Lab. Invest. 70: 176-182.

Sarin S., Cheatle T.R., Colridge Smith P.D., Scurr J.H. (1991) Disease mechanisms in venous ulceration. Br. J. Hosp. Med. 45: 303-305.

Sarret Y., Woodley D.T., Goldberg G.S., Kronberger a., Wynn K.C. (1992a) Constitutive synthesis of a 92kDa keratinocyte derived type IV collagenase is enhanced by type I collagen and decreased by type IV collagen matrices. J. Invest. Dermatol. 99: 836-841.

Sarret Y., Woodley D.T., Grigsby K., Wynn K., O'Keefe E.J. (1992b) Human keratinocyte locomotion: the effect of selected cytokines. J. Invest. Dermatol. 98: 12-16.

Schmid P., Cox D., Bilbe G., McMaster G., Morrison C., Stahelin H., Luscher N., Seiler W. (1993) TGF-βs and TGF-β type II receptor in human epidermis: Differential expression in acute and chronic skin wounds. J. Pathol. 171: 191-197.

Schreiber A.B., Winkler M.E., Derynck R. (1986) Transforming growth factor α: A more potent angiogenic mediator than epidermal growth factor. Science. 232: 1250-3.

Schultz G., Bennett N., Rotatori S., Macaully S., Moser M. (1993) EGF and TGFα: analysis of wound fluids and effects of exogenous factors on wound healing. J. Cell Biochem. 17E (Suppli), 109.

Schultz G., Rotatori D. & Clark W. (1991) EGF and TGF-α in wound healing and repair. J. Cell Biochem. 45: 346-352.

Schumacher J. & Hanselka D.V. (1989) Skin grafting of the Horse. Vet. Clin. North Amer.: Equine practice 5: 591-614.

Senior R.M., Griffin G.L. & Mecham R.P. (1980) Chemotactic activity of elastin-derived peptides. J. Clin. Invest. 66: 859-862.

Senior R.M., Skozen W.F. & Griffin G.L., Wilner G.D. (1986) Effects of fibrinogen derivatives upon the inflammatory response. J. Clin. Invest. 77: 1014-1019.

Seppa H., Grotendorst G., Seppa S., Schiffman E., Martin G.R. (1982) Platelet- derived growth factor is chemotactic for fibroblasts. J. Cell Biol. 92: 584-588.

Shah M., Forman D.M., Ferguson M.W.J. (1992) Control of scarring in adult wounds by neutralising antibody to TGFβ. Lancet 339: 213-214.

Shah M., Forman D.M., Ferguson M.W.J. (1994) Neutralising antibody to $TGF\beta_{1,2}$ reduces cutaneous scarring in adult rodents. J. Cell Science 107: 1137-1157.

Shami S.K., Shields D.A., Scurr J.H., Coleridge Smith P.D. (1992) Leg ulceration in venous disease. Postgrad. Med. J. 68: 779-785.

Shapiro S.D., Griffin G.L., Gilbert D.J. Jenkins N.A., Copeland N.G., Welgus H.G., Senior R,M., Ley T.J. (1992) Molecular cloning chromosomal localisation and bacterial expression of a murine macrophage metalloelastase. J. Biol. Chem. 267: 4664-4671.

Shimokado K., Raines E.W., Madtes D.K., Barratt T.B., Benditt E.P., Ross R. (1985) A significant part of macrophage-derived growth factor consists of at least two forms of platelet derived growth factor. Cell 43: 277-286.

Shoshan S. (1981) Wound healing. In: International review of connective tissue research, vol 9, (D.A. Hall & D.S. Jackson eds.) pp 1-26, Academic, New York.

Sieweke M.H., Thompson N.L., Sporn M.B., Bissel M.J. (1990) Mediation of wound related Rous Sarcoma virus tumorigenesis by TGFβ. Science 248: 1656

Silver I.A. (1982) Basic physiology of wound healing in the horse. Equine vet. J. 14: 7-15.

Simon B. (1988) Treatment of wounds. In emergency medicine: Concepts and clinical practice, ed 2 St. Louis, CV Mosby. pp 363-373.

Simpson D. & Ross R. (1972) The neutrophilic leukocyte in wound repair. A study with anti-neutrophil serum. J. Clin. Invest. 51: 2009-2023.

Singer I.I., Kawka D.W., Kazazis D.M., Clark R.A.F. (1984) *In vivo* codistribution of fibronectin and actin fibres in granulation tissue: Immunofluorescence and electron microscope studies of the fibronexus at the myofibroblast surface. J. Cell Biol. 98: 2091-2106.

Slavin J., Hunt J.A., Nash J.R., Williams D.F., Kingsnorth A.N. (1992) Recombinant basic fibroblast growth factor in red blood cell ghosts accelerates incisional wound healing. Br. J. Surg. 79: 918-921.

Sporn M. B.& Roberts A.B. (1986) Peptide growth factors and inflammation, tissue repair and cancer. J. Clin. Invest. 78: 329-332.

Sporn M.B. & Roberts A.B. (1990) TGFβ: Problems and prospects. Cell Regul. 1: 875-882.

Sporn M.B., Roberts A.B., Shull J.H., Smith J.M., Ward J.M. (1983) Polypeptide transforming growth factors isolated from bovine sources and used for wound healing *in vivo*. Science 219: 1329-1330.

Sporn M.B., Roberts A.B., Wakefield L.M., Assoian R.K. (1986) Transforming growth factor-β: Biological function and chemical structure. Science 233: 532-4.

Sporn M.B., Roberts A.B., Wakefield L.M., DeCromgrugghe B. (1987) Some recent advances in the chemistry and biology of TGFβ. J. Cell Biol. 105: 1039-45.

Stashak T.S. (1984) The practice of large animial surgery. Jennings P.B. (Ed), W.B. Saunders Philadelphia.

Stegman H. & Stadler K. (1967) Determination of hydroxyproline. Clin. Chem. Acta. 18: 267-273.

Steinberg B.M., Smith K., Colozzo M., Pollack R. (1980) Establishment and transformation diminish the ability of fibroblasts to contract a native collagen gel. J. Cell Biol. 87: 304-308.

Stenn K.S., Madri J.A. & Roll F.J. (1979) Migrating epidermis produces AB_2 collagen and requires continued collagen synthesis for movement. Nature 277; 229-232.

Strongin A.Y., Collier I., Bannikov G., Marmer B.L., Grant G.A., Goldberg G.I. (1995) Mechanism of cell surface activation of 72kDa type IV collagenase. J. Biol. Chem. 270:5331-5228.

Swain S.F. (1980) Surgery of traumatised skin. W.B. Saunders, Philadelphia.

Swerlick R.A., Garcia-Gonzolez E., Kubota Y., Xu Y., Lawley T.J. (1991) Studies of the modulation of MHC antigen and cell adhesion molecule expression on human dermal microvascular endothelial cells. J. Invest. Dermatol. 97: 190-196.

Swerlick R.A. & Lawley T.J. (1993) Role of microvascular endothelial cells in inflammation. J.Invest Dermatol. 100: 111s-115s.

Tarwid J.N., Fretz P.B., Clark E.G. (1985) Equine sarcoids. A study with emphasis on pathological diagnosis. Compend. Cont. Educ. Pract. Vet. 7: 293-300.

Terranova V.P., Diflorio R., Lyall R.M., Hic S., Fressal R., Macaig T. (1985) Human endothelial cells are chemotactic to endothelial cell growth factor and heparin. J. Cell Biol. 101: 2330-2334.

Trenfield K., Spadbrow P.B., Vanselow B. (1985) Sequences of papilloma virus DNA in equine sarcoids. Equine Vet. J. 17: 449-452.

Trott A.. (1991) Surface injury and wound healing. In wounds and lacerations: Emergency care and closure. Mosby Year Book. St Louis. pp 12-23.

Tseng S.C.G., Savion N., Stern R., Gospodarowicz D. (1982) Fibroblast growth factor modulates synthesis of collagen in cultured fibroblasts.122: 355-360.

Van-Obberghen-Schilling E., Roche N.S., Flanders K.C. Sporn M.B., Roberts A.B. (1988) Transforming growth factor-β1 positively regulates its own expression in normal and transformed cells. J. Biol. Chem. 263: 7741-7746.

Vlodavsky I., Folkman J., Sullivan R., Fridman R., Ishai-michaeli R., Sasse J., Klagsbrun M. (1987a) Endothelial cell derived basic fibroblast growth factor: Synthesis and deposition into subendothelial extracellular matrix. Proc. Natl. Acad. Sci. USA.84: 2292-6.

Vlodavsky I., Fuks Z., Ishai-Micheali R., Bashkin P., Levi E., Korner G., Bar-Shavit R., Klagsbrun M. (1987b) Extracellular matrix -resident basic fibroblast growth factor: implication control of angiogenesis. J. Cell Biochem. 42: 167-176.

Voss J.L. (1969) Transmission of equine sarcoid. Am. J. Vet. Res. 30: 183-191.

Wachtfogel Y.T., Abrams W., Kucich U., Weinbaum G., Schapira, M., Colman R.W. (1988) Fibronectin degradation products containing the cytoadhesive tetrapeptide stimulate human neutrophil degranulation. J. Clin. Invest. 81: 1310-1316.

Wahl S.M., Hunt D.A., wakefield L.M., McCartney-Francis N., Wahl L.M., Roberts A.B., Sporn M.B. (1987) Transforming growth factor type-β induces monocyte chemotaxis and growth factor production. Proc. Natl. Acad. Sci. USA 84: 5788-92.

Wahl S.M. & Wahl L.M. (1985) Regulation of macrophage collagenase, prostaglandin and fibroblast activating-factor production by anti- inflammatory agents: Different regulatory mechanisms for tissue injury and repair. Cell Immunol. 92: 302-312.

Wakefield L.M., Smith D.M., Flanders K.C., Sporn M.B. (1987) Characterisation of a latent form of Transforming growth factor-β secreted by human platelets. J. Biol. Chem. 11A (suppli) 46.

Wakefield L.M., Smith D.M., Flanders K.C., Sporn M.B. (1988) Latent transforming growth factorβ from human platelets, a high molecular weight complex containing precursor sequences. J. Biol. Chem. 263: 7646-7654.

Walker L.N., Bowen-Pope D.F., Ross R., Reidy M.A. (1986) Production of PDGF-like molecules by cultured arterial smooth muscle cells accompanies proliferation after arterial injury. Proc. Natl. Acad. Sci. USA 83: 7311-5.

Ward R.V., Atkinson S.J., Slocombe P.M., Dochery A.J.P., Reynolds J.J., Murphy G. (1991a) Tissue inhibitor of MMP 2 inhibits the activation of 72kDa progelatinase by fibroblast membranes. Biochem. Biophys. Acta. 1079: 242-246.

Ward R.V., Hembry R.M., Reynolds J.J., Murphy G. (1991b) The purification of Tissue inhibitor of metalloproteinases-2 from its 72 kDa progelatinase complex. Demonstration of the biochemical similarities of tissue inhibitor of Metalloproteinases-2 and tissue inhibitor of Metalloproteinases-1. Biochem. J. 278:179-187.

Welch M.P., Odland G.F. & Clark R.A.F. (1990) Temporal relationship of F-actin bundle formation; collagen and fibronectin matrix assembly and fibronectin receptor expression to wound contraction. J. Cell Biol. 110: 133-145.

Wenner A., Leu H.J., Spycher M., Brunner U. (1980) Ultrastructural changes in capillaries in chronic venous insufficiency. Expl. Cell Biol. 48: 1-14.

Werb Z. & Gordon S. (1975a) Secretion of a specific collagenase by stimulated macrophages. J.Exp. Med. 142: 346-360.

Werb Z. & Gordon S. (1975b) Elastase secretion by stimulated macrophages. J. Exp. Med. 142: 361-377.

Werb Z., Mainardi C.L., Vater C.A. & Harris E.D. (1977) Endogenous activation of latent collagenase by rhuematoid synovial cells: Evidence for a role of plasminogen activator. N. Engl. J. Med. 296: 1017-1023.

Werb Z., Temble P.M., Behrendtsen O., Crowley E., Damsky C.H. (1989) Signal transduction through the fibronectin receptor induces collagenase and stromelysin gene expression. J. Cell Biol. 109: 877-889.

Werner S., Peters K.G., Longaker M.T., Fuller-Pace F., Banda M.J., Williams L.T. (1992) Large induction of keratinocyte growth factor expression in the dermis during wound healing. Proc. Natl. Acad. Sci. USA 89: 6896-6900.

Westermark B. & Blomquist E. (1980) Stimulation of fibroblast migration by epidermal growth factor. Cell Biol. Int. Rep. 4: 649-54.

Whitman S.E., Murphy G., Angel P., Rahmsdorf H.J., Smith B.L., Lyons A., Harris T.J.R., Reynolds J.J., Docherty A.J.P. (1986) Comparison of human stromelysin and collagenase by cloning and sequence analysis. Biochem J. 240: 913-916.

Wilhelm S.M., Collier I.E., Marmer B.L., Eisen A.Z., Grant G.A., Goldberg G.I. (1989) SV40-transformed human lung fibroblasts secrete a 92kD type IV collagenase which is identical to that secreted by normal human macrophages. J. Biol. Chem. 264:17213-17221.

Wilkinson L.S., Bunker C., Edwards J.C.W., Scurr J.H., Coleridge-Smith P.D. (1993) Leucocytes: Their role in etiopathogenesis of skin damage in venous disease. J. Vasc. Surg. 17: 669-675.

Wilkner N.E., Perichitte K.A., Baskin J.B., Nielson L.D. Clark R.A.F. (1988) Transforming growth factor β stimulates the expression of fibronectin by human keratinocytes. J. Invest. Dermatol. 91: 207-12.

Williams I.F., Heaton A., McCullagh K.G. (1982) Connective tissue composition of the equine sarcoid. Equine Vet. J. 14: 305-310.

Wilson C.L. & Schwrazbauer J.E. (1992) The alternatively spliced V region contributes to the differential incorporation of plasma and cellular fibronectin into fibrin clots. J. Cell Biol. 119:923-933.

Wiseman D.M., Polverini P.J., Kamp D.W., Leibovich S.J. (1988) Transforming growth factor β is chemotactic for human monocytes and induces their expression of angiognenic activity. Biochem. Biophys. Res. Commun. 157: 793-800.

Woessner J.F. Jnr. (1991) Matrix metalloproteinases and their inhibitors in connective tissue remodelling. FASEB J. 5: 2145-2154.

Wood A.L., Spadbrow P.B. (1985) Transmission of cultured equine fibroblasts with a bovine papilloma virus. Res. Vet. Sci. 38: 241-242.

Woodley D.T., O'Keefe E.J., Prunieras M. (1985) Cutaneous wound healing: A model for cell matrix interactions. J. Am. Acad. Dermatol. 12: 420-433.

Wysocki A.B. & Grinnell F. (1990) Fibronectin profiles in normal and chronic wound fluid. Lab. Invest. 63: 825-831.

Wysocki A.B., Staiano-Coico L. & Grinnell F. (1991) Proteolytic enzyme cascade over-expressed in chronic wounds. J. Cell Biol. 115: 137a.

Wysocki A.B., Staiano-Coico L. & Grinnell F. (1993) Wound fluid from chronic leg ulcers contains elevated levels of MMP2 and MMP9. J. Invest. Dermatol. 101: 64-68.

Yamada K.M. (1989) Fibronectins: Structure, function and receptors. Curr. Opin. Cell. Biol. 1: 956-963.

Yamada K.M. & Weston J.A. (1974) Isolation of a major cell surface glycoprotein from fibroblasts. Proc.Natl. Acad. Sci USA 71: 3492-3496.

Yamaguchi M., Mann D.M., Ruoslahti E. (1990) Negative regulation of transforming growth factor-β by the proteoglycan decorin. Nature 346: 281-284.

Zeitz M., Ruis-Torres A., Merker H.J. (1978) Collagen metabolism in granulating wounds of rat skin. Arch. Dermatol. Res. 263: 207-214.

APPENDIX A

SDS gel electorphoresis - Separating gel preparation (7.5%)

Distilled water	4.85ml
1.5M Tris-HCl pH 8.8	2.5ml
10% (w/v) SDS stock	100µl
Acrylamide/Bis (30%) stock	2.5 ml
10% ammonium persulphate (APS)	50µl
TEMED	5µl

10% APS was prepared fresh daily

APPENDIX B

SDS gel electorphoresis - Stacking gel preparation (4%)

Distilled water	6.1ml
0.5M Tris-HCl pH 6.8	2.5ml
10% (w/v) SDS stock	100µl
Acrylamide/Bis (30%) stock	1.3 ml
10% ammonium persulphate (APS)	50µl
TEMED	10µl

10% APS was prepared fresh daily

APPENDIX C

Zymography - Resolving gel preparation (7.5%)

Distilled water	2.4ml
1.5M Tris-HCl pH 8.8	2.5ml
20% (w/v) SDS stock	50µl
Acrylamide/Bis (30%) stock	2.5 ml
Gelatin stock (1%)	2.5ml
10% ammonium persulphate (APS)	100µl
TEMED	7.5µl

10% APS was prepared fresh daily

APPENDIX D

Zymography - Stacking gel preparation (4%)

Distilled water	6.1ml
0.5M Tris-HCl pH 6.8	2.5ml
20% (w/v) SDS stock	50µl
Acrylamide/Bis (30%) stock	1.3 ml
10% ammonium persulphate (APS)	100µl
TEMED	5µl

10% APS was prepared fresh daily

PUBLICATION IN SUPPORT OF THIS THESIS

Effect of growth factors on the characteristics of cells associated with equine wound healing and sarcoid formation

C. A. COCHRANE, BSc(HONS); K. L. FREEMAN, BSc; D. C. KNOTTENBELT, BVM & S, DVM & S, MRCVS

Wound healing in equidae is delayed and more complicated than in other species. These complications arise from a condition known as exuberant granulation tissue formation. The lower limb of the horse is frequently slower to heal than other parts of the body and has a particular tendency to produce excess (exuberant) granulation tissue. Sarcoids are tumor-like lesions of the skin which often appear at the site of wounds. This study compared the growth characteristics of the sarcoid and granulation tissue-derived cells with normal dermal fibroblasts grown from primary cell cultures. All three cell types had distinct morphologic differences. Growth rate studies showed that the sarcoid and granulation tissue-derived cells grew at a slower rate than the normal cells. The addition of the growth factor epidermal growth factor, acidic fibroblast growth factor, and basic fibroblast growth factor selectively stimulated the replication of normal and sarcoid-derived cells but inhibited the growth of granulation tissue-derived cells. In contrast, transforming growth factor-β was not preferentially inhibitory for the granulation tissue-derived cells. The addition of growth factors to the medium also produced distinct alterations in the morphology of the cells. **(WOUND REP REG 1996;4:58-65)**

Skin is a complex organ that cannot regenerate, and therefore it must be repaired after injury.[1] In normal circumstances the healing process is achieved by wound debridement, protection of the wound bed with a highly vascular and resistant tissue (granulation tissue), reduction of the wound size by contraction, and permanent surface protection by epithelial migration. However, this is not always the case in horses, where wound healing is commonly delayed and is more complicated than in other species.[2-4]

From the Department of Veterinary Clinical Science, Equine Studies, University of Liverpool, Veterinary Field Station, Leahurst, Neston, South Wirral, United Kingdom.

Reprint requests: C. A. Cochrane, BSc, Department of Veterinary Clinical Science, University of Liverpool, Leahurst, Neston, South Wirral, L64 7TE, United Kingdom.

1067-1927/96 $5.00 + 0 36/1/71219

DMEM	Dulbecco's modified Eagle's medium
EGF	Epidermal growth factor
FGF	Fibroblast growth factor
TGF-β	Transforming growth factor-β

Horses tend to produce excessive granulation tissue especially in the lower limb extremities.[4-8] Unsightly exuberant granulation tissue is formed which delays or halts wound healing and may lead to secondary complications, such as bacterial infections.

The most common cutaneous tumors in horses are sarcoids, which are fibroblastic wart-like lesions. Sarcoids are not considered to be malignant but are often invasive and recurrent. Sarcoids may occur on any part of the body, alone or in clusters, but the head, ventral abdomen, and limbs are most affected.[9,10] It is not uncommon for sarcoids to appear at the site of wounds.[11] Their appearance can vary, and they are classified into six types: occult, verrucous, nodular, fibroblastic, mixed, and malevolent.[12,13] These tumors show linear or focal dermal

thickening and are pale in color. Their texture is due to fibroblast proliferation and the relatively poor vascularization of the tissue.[14] Histopathologic confirmation of the diagnosis is required to distinguish fibroblastic sarcoids from exuberant granulation tissue, other rare mesenchymal tumors, or other infectious and noninfectious granulomas.[15-17] Considerable circumstantial evidence exists that sarcoids are caused by viruses closely related or identical to the bovine papilloma viruses.[18-20]

Previous investigations indicated that no significant difference existed between the proliferation rates of fibroblast cells cultured from the limb and other sites in the equine body or fibroblast cells cultured from granulation tissue from healing and nonhealing wounds.[3] Therefore the current study was undertaken to investigate the response of these cells and fibroblast cells of sarcoid origin to cell growth factors.

MATERIALS AND METHODS

Source of tissue

Dermal tissue was obtained post mortem from horses and a pony which had been killed for other reasons. These horses were between the ages of 5 and 13 years old and all Hackney, Thoroughbred, or Bay Roan geldings. Six different anatomic sites were sampled, as follows: eyelid, axilla, groin, medial thigh, ventral midline, and limb (fore, mid-dorsal cannon). Similar tissue was taken from horses with slow-healing granulating wounds with exuberant granulation tissue during normal surgical debridement before skin grafting. This group of horses included a yearling Thoroughbred male, a 12-year-old Thoroughbred mare, two 12-year-old Arabian mares, and a 6-year-old Thoroughbred gelding. Samples were taken from superficial, middle, and deep sites within the exuberant granulation tissue. The age of the granulation tissue ranged from 3 to 8 weeks. Horses with sarcoids which were being killed also had dermal tissue removed. Four types of sarcoid were investigated: verrucous, nodular, fibroblastic, and mixed. The horses in this group were between 7 and 14 years old. Both geldings and mares were sampled from Welsh, Hunter, and Thoroughbred breeds.

Tissue samples were immediately transferred to a dish of Hank's balanced salt solution (Gibco, Paisely, Scotland) and washed. The tissues were cut into 3 to 5 mm^2 pieces and placed into 25 cm^2 tissue culture flasks (Nunc, A/S, Roskilde, Denmark) containing Dulbecco's modified Eagle's medium (DMEM; Gibco), supplemented with 10% fetal calf serum (Sigma Chemical Co., Poole, Dorset, United Kingdom), HEPES buffer 20 mmol/L, gentamicin 100 µg/ml, and amphotericin B 0.5 µg/ml (Gibco). The flasks were incubated in a 5% carbon dioxide in air environment at 37° C.

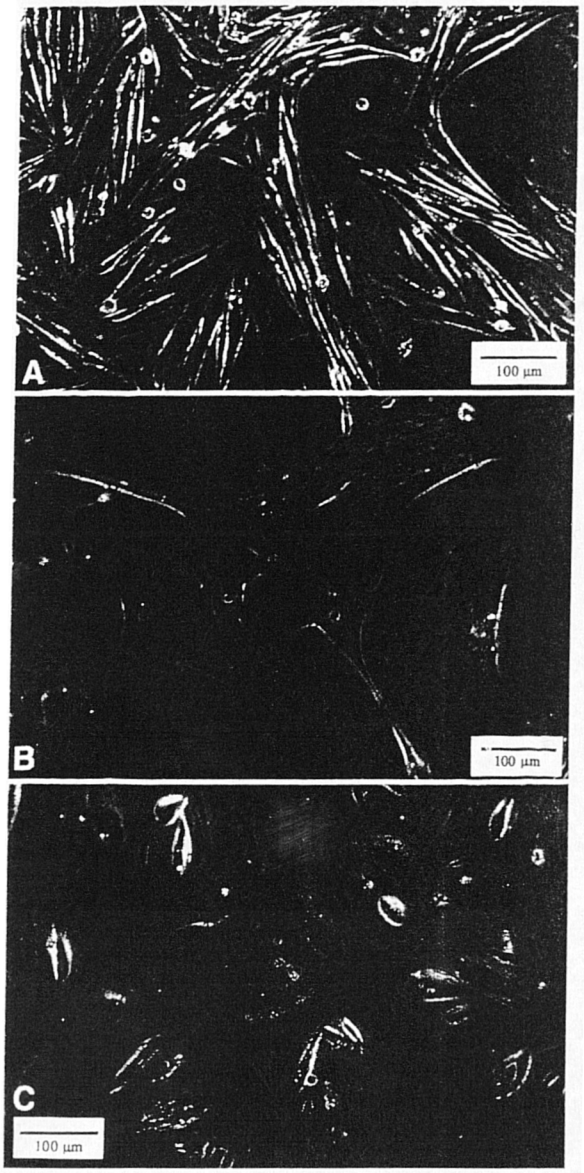


Figure 1 Phase contrast photomicrographs of cultured cells. **A,** Granulation tissue–derived fibroblasts. **B,** Fibroblastic-type sarcoid cells. **C,** Nodular-type sarcoid cells. Original magnification ×100.

Readiness for subculturing the cells was determined by the extent of fibroblast cell outgrowth (5 to 10 days). Cells were successively replated in a 1:4 split ratio to passage 3-8 for experimental use.

Table 1. Descriptive changes in cell morphology with the addition of growth factors

	Growth factor treatment				
Cell type	Untreated	EGF	FGFa	FGFb	TGF-β
Normal fibroblasts	Spindle shaped characteristic morphology	Spindle shaped as untreated cells	Spindle shaped as untreated cells	Thinner spindle shaped Figure 2, *B*)	Flattened enlarged cells Figure 2, *A*
Granulation tissue–derived fibroblasts	Spread out cuboidal appearance (Figure 1, *A*)	Large expanded cells with spiky edges (Figure 2, *G*)	Irregular shaped cells (Figure 2, *D*	Thinner spindle shaped Figure 2, *A*	Flattened enlarged cells (Figure 2, *A*)
Sarcoid-derived fibroblasts	Fibroblastic, verrucose, and mixed: large, spiky edges (Figure 1, *B*) and nodular: smaller, rounder (Figure 1, *C*)	Flatter, spread out, and larger cells (Figure 2, *F*)	Large flatter cells Figure 2, *E*	Shorter compact spindles (Figure 2, *C*)	Flattened, enlarged cells Figure 2, *A*

Cell proliferation analysis

Normal fibroblasts from all six sites, cells derived from exuberant granulation tissue, and those from verrucous, nodular, fibroblastic, and mixed sarcoid tumors were harvested from stock dishes and pooled. Single-cell suspensions of these cells were plated onto 35 mm plastic culture dishes (Nunc) in duplicate at 6 × 10^4 cells/dish in 1 ml DMEM supplemented with 2.5% fetal calf serum and 1% penicillin-streptomycin, HEPES buffer 20 mmol/L (Gibco). Cells were left to attach for 6 hours, after which the plating medium was replaced with "experimental" medium: DMEM, 1% penicillin-streptomycin, HEPES buffer 20 mmol/L, 2.5% fetal calf serum containing either 10 ng/ml epidermal growth factor (EGF; Sigma), 10 ng/ml fibroblast growth factor (FGF) a and FGFb or 1 ng/ml transforming growth factor-β (TGF-β, R&D Systems, Abingdon, Oxon, United Kingdom). Medium was changed every 2 days. Cells from duplicate dishes were released by treatment with 0.5% trypsin/EDTA solution (Gibco) for 5 minutes at 37° C and counted in a Neubauer counting chamber, every 5 days up to and including day 20. All of the results were expressed as the mean ± standard error of the mean (SEM). The statistical significance of the results was assessed with Student's unpaired t test, and a p value of < 0.05 was considered to be significant.

Photomicroscopy

Photomicrographs were taken 48 hours after subculturing by means of an Olympus CK2 inverted microscope with a photomicrograph attachment PM10-AK-2 and an Olympus C35 DA/2 camera (Olympus Optical, Hamburg, Germany). A Kodak Ektachrome 64T tungsten film (Eastman Kodak Co., Rochester, N.Y.) was used from which black and white prints were made.

RESULTS

Morphology

The fibroblasts from the different primary cultures showed characteristic morphologies (Table 1). The cells from normal tissue showed the typical spindle-shaped morphology, whereas the granulation tissue–derived fibroblasts had a spread out and cuboidal appearance (Figure 1, *A*). Cells from the fibroblastic mixed and verrucous type sarcoid tissue were large, spread cells with spiky edges (Figure 1, *B*) but the cells derived from nodular sarcoid tissue (Figure 1, *C*) appeared smaller and rounder.

Addition of growth factors to the medium had an effect on the morphology of all cell types (Table 1). In media containing TGF-β the cells underwent distinctive changes in morphology, becoming flattened and enlarged with an apparent increased cytoplasmic/nuclear ratio, although this was not quantified (Figure 2, *A*). The normal and granulation tissue–derived fibroblasts (Figure 2, *B*) adopted a thinner spindle-shaped morphology with FGFb, whereas the sarcoid-derived cells produced much shorter compact spindles (Figure 2, *C*). Granulation tissue–derived fibroblasts grown in media containing FGFa (Figure 2, *D*) had irregular shaped cells but the sarcoid-derived cells transformed to larger flattened cells similar to the morphologic changes that occurred with TGF-β (Figure 2, *E*). The normal cell morphology remained the same.

Media supplemented with EGF had little effect on the morphology of normal cells. However, the sarcoid cell morphology was altered to a more flatter, spread, and larger cell shape (Figure 2, *F*) in comparison with the control cells. In contrast, EGF-treated granulation tissue–derived cells altered their morphology similar to the morphology of the fibroblastic sarcoid cells, being larger, expanded cells with spiky edges

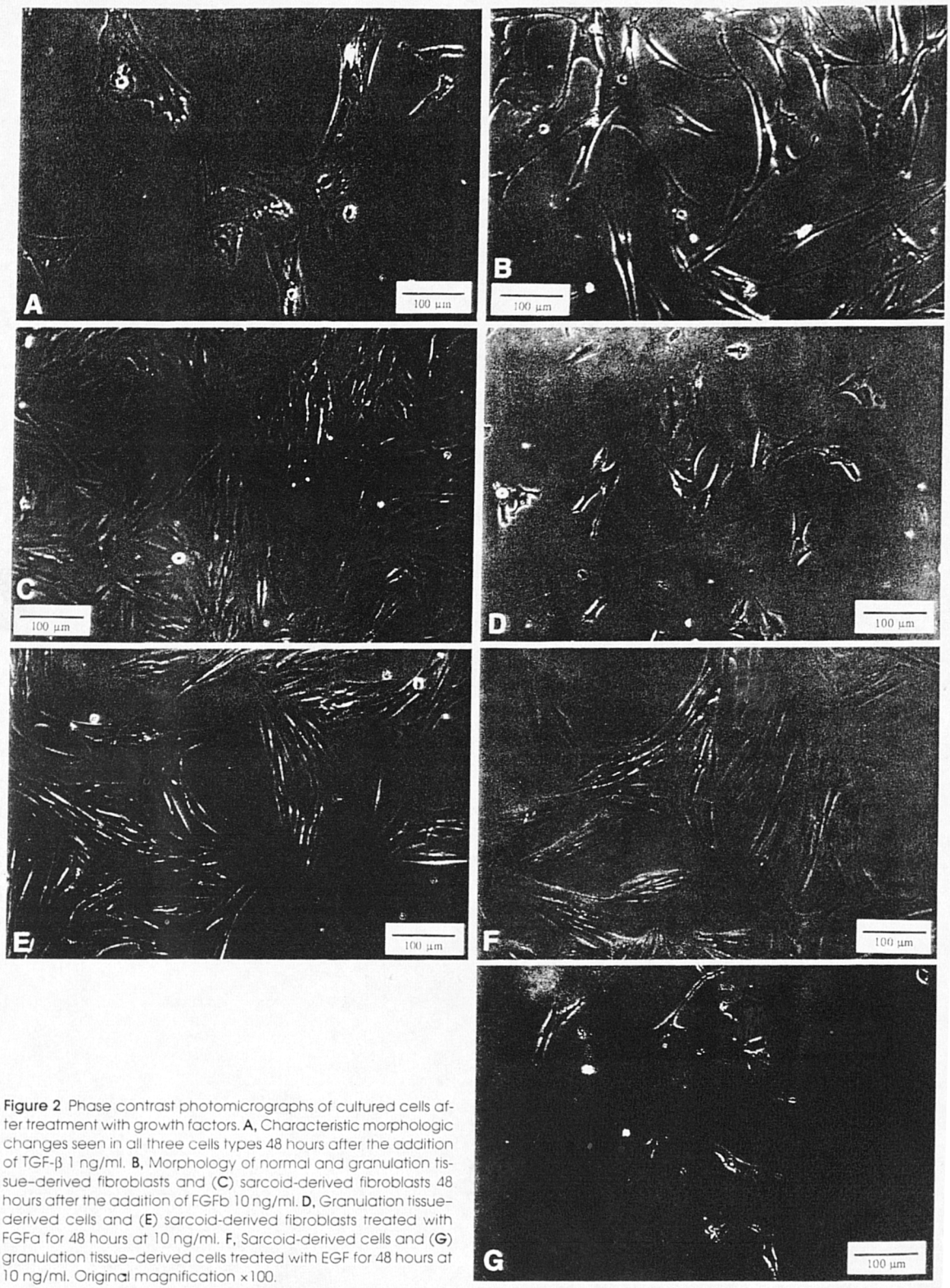


Figure 2 Phase contrast photomicrographs of cultured cells after treatment with growth factors. **A,** Characteristic morphologic changes seen in all three cells types 48 hours after the addition of TGF-β 1 ng/ml. **B,** Morphology of normal and granulation tissue–derived fibroblasts and (**C**) sarcoid-derived fibroblasts 48 hours after the addition of FGFb 10 ng/ml. **D,** Granulation tissue–derived cells and (**E**) sarcoid-derived fibroblasts treated with FGFa for 48 hours at 10 ng/ml. **F,** Sarcoid-derived cells and (**G**) granulation tissue–derived cells treated with EGF for 48 hours at 10 ng/ml. Original magnification ×100.

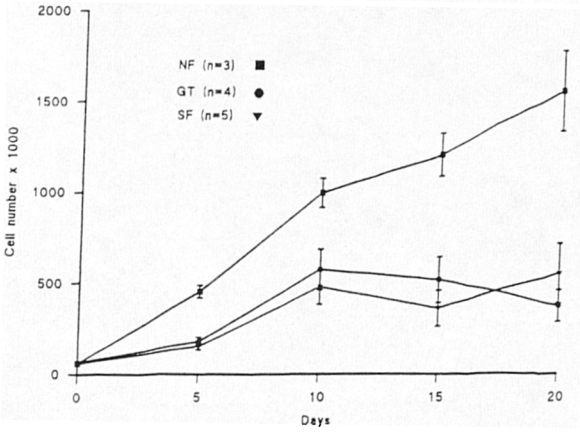


Figure 3 Growth rates of normal (*NF*), granulation tissue (*GT*), and sarcoid-derived (*SF*) fibroblasts. Cells were grown for 20 days and counted every 5 days. Each value indicates mean ± standard error of the mean

(Figure 2, *G*). These morphologic differences were seen each time the growth factors were added to the cultures. There appeared to be no alteration in these morphologic changes as the cells neared confluence.

Growth rates

No significant difference was found in growth rate between the normal fibroblasts cells from the limb and other sites of the body under standard culture conditions. Similarly, no significant difference was seen in the growth rates among the four types of sarcoid cells used in these investigations. Therefore the data for both the normal cells and the sarcoid cells were pooled.

Comparison of the rate of growth of normal cells, sarcoid-derived cells, and the granulation tissue–derived cells are shown in Figure 3. A statistically significant difference at day 5 was seen between the normal and the granulation tissue cells ($p < 0.001$) as well as a statistically significant difference between the normal and the sarcoid cells ($p < 0.0001$). At day 10, a similar observation occurred with a statistically significant difference ($p < 0.05$) between normal and granulation tissue–derived cells and a statistically significant difference ($p < 0.005$) between normal and sarcoid-derived cells. The growth rates for days 15 and 20 show that a statistically significant difference ($p < 0.01$) between normal and granulation tissue–derived cells and a significant difference ($p < 0.001$) between normal cells and sarcoid-derived cells continued on both days. No significant difference was observed between the granulation tissue–derived and the sarcoid-derived cells on any of the 5 counting days.

Growth factor effect on growth rates

The effect of EGF on the cell number of all three cell types is present in Figure 4, *A*. On day 5, no significant difference was observed between the effect on the normal cells and the other cells with EGF treatment. However, the sarcoid cells had proliferated at a higher rate than either the normal or the granulation tissue cells treated with EGF. The granulation tissue–derived cells had the lowest proliferation rate of all three cell types.

By day 10, the normal fibroblasts treated with EGF had increased in cell numbers with respect to EGF-treated granulation tissue cells or sarcoid cells. The EGF-treated granulation tissue cells had decreased in number and the sarcoid cells had increased, but the cultures had fewer cells than the EGF-treated normal cells. The normal treated cells showed a significantly higher cell number ($p < 0.005$) than the granulation tissue–derived cells, but no difference between the normal and sarcoid-derived EGF-treated cells. Also, no significant difference was observed between the nontreated sarcoid and granulation tissue–derived cells. EGF had and inhibitory effect on the granulation tissue–derived cells at day 15. The normal cells treated with EGF showed a greater response to this growth factor than the granulation tissue–derived cells ($p < 0.002$). Likewise the sarcoid cells showed a similar response with a significant difference ($p < 0.05$) from the granulation tissue cells. On the final day of the experiment (day 20), a significant difference ($p < 0.001$) was observed in cell number between the normal and granulation tissue cells treated with EGF. A greater stimulatory effect was observed on normal cells with EGF than on the sarcoid-derived cells ($p < 0.002$). A greater stimulatory effect was observed on sarcoid-derived cells than on the granulation tissue–derived cells ($p < 0.05$) with the addition of EGF.

The addition of FGFa (Figure 4, *B*) to the media of normal cells increased their cell numbers at day 10 but at a lower rate than cells treated with EGF. However, the addition of FGFa to the media of granulation tissue-derived cells appeared to inhibit the proliferation rate. A significant difference ($p < 0.005$) was observed between the cell number of granulation tissue and normal cells treated with FGFa. At day 15, treatment with FGFa had stimulated the normal cells to proliferate but had the opposite effect on the granulation tissue–derived cells ($p < 0.0001$). At the end of the experiment (day 20), there was a significant difference ($p < 0.001$) in cell number between normal and granulation tissue cells treated with FGFa. Cell number increased to a greater extent in the normal cells

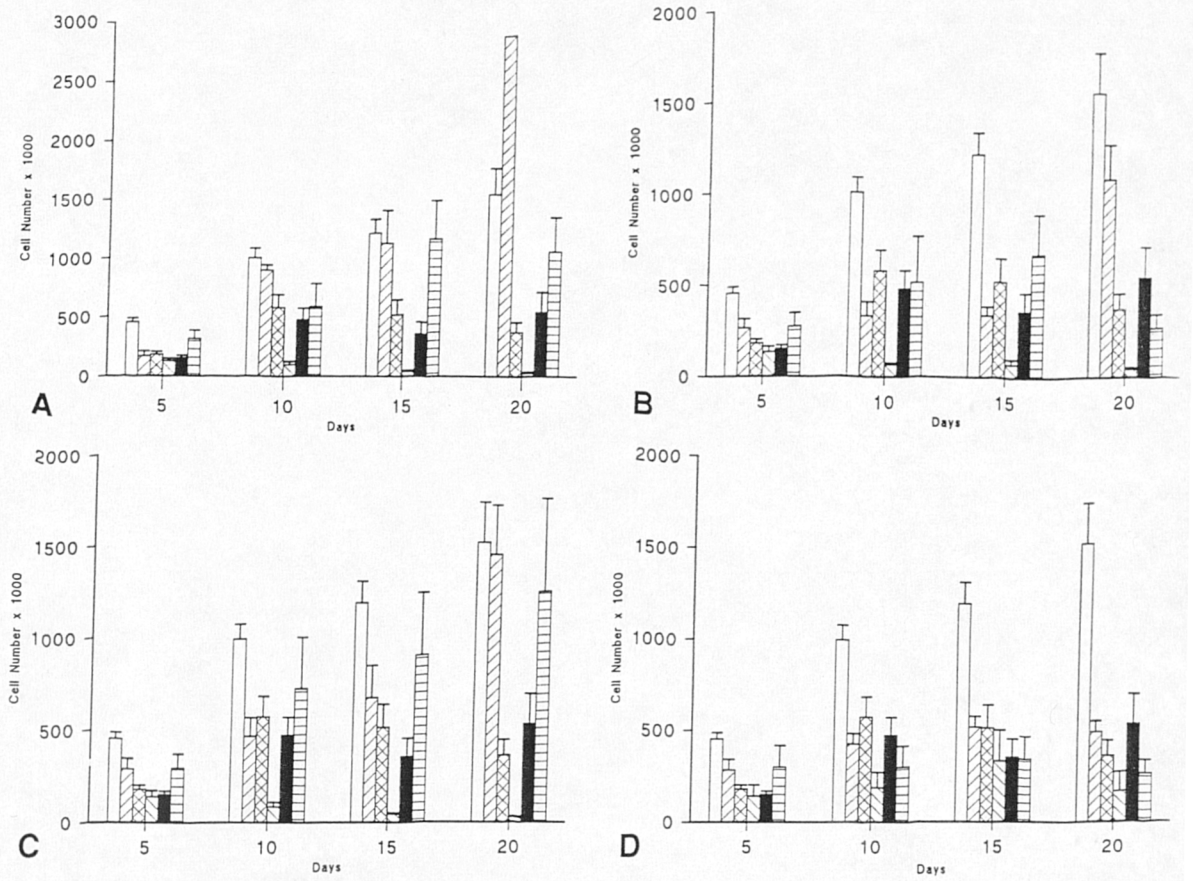


Figure 4 Growth rates of normal ($n = 3$), granulation tissue ($n = 4$), and sarcoid-derived ($n = 5$) fibroblasts treated with EGF, FGFa, FGFb at 10 ng/ml, or TGF-β 1 ng/ml for 20 days and counted every 5 days. **A**, Effect of the addition of EGF. **B**, FGFa. **C**, FGFb. **D**, TGF-β. *Bars* represent the following: *open*, untreated control cells; *striped (/)*, treated normal fibroblasts; *crossed (x)*, untreated granulation tissue–derived cells; *striped (\)*, treated granulation tissue–derived cells; *solid*, control sarcoid-derived cells; *dashed (--)*, treated sarcoid-derived cells.

treated with FGFa ($p < 0.001$) than with the sarcoid-derived cells.

When the cells were treated with FGFb at day 10 (Figure 4, *C*), cell numbers increased with all cell types except the granulation tissue–derived cells which decreased in cell numbers. Normal cells treated with FGFb showed a significantly higher cell number ($p < 0.02$) than the treated granulation tissue–derived cells.

In contrast to the other growth factors, at day 10 TGF-β treatment allowed cell growth for all three cell types and a significant difference was observed between the treated normal and granulation tissue cells ($p < 0.05$).

The granulation tissue–derived cells treated with TGF-β continued to grow at day 15 (Figure 4, *D*), whereas the other growth factors had an inhibitory effect on these cells.

On the final day of the experiment (day 20), there was a significant difference ($p < 0.001$) in growth rates between the normal and granulation tissue cells treated with EGF, FGFa, and FGFb and a difference of $p < 0.05$ with the addition of TGF-β (Figure 4, *D*). A greater stimulatory effect occurred on normal cells with EGF than on sarcoid-derived cells ($p < 0.002$). A similar effect was seen with FGFa ($p < 0.001$) and TGF-β ($p < 0.05$). A greater stimulatory effect was observed on sarcoid-derived cells than on the granulation tissue–derived cells ($p < 0.05$) with the addition of EGF.

DISCUSSION

This study showed a difference in cell morphology and growth rate characteristics among the normal fibroblast, sarcoid-derived, and granulation tissue–derived

cells. The slow growth rates of granulation tissue and sarcoid-derived cells could be due to cell to cell contact inhibition occurring because these cells are much larger than normal fibroblasts and therefore the confluence level was achieved much sooner and with a lower number of cells. The morphology of the sarcoid- and granulation tissue–derived cells differed from normal fibroblasts, and it has been suggested that histopathologic confirmation of the tissue types is necessary to distinguish sarcoid tissue from granulation tissue.[15-17]

Because of the clinical observations made with exuberant granulation tissue on the lower limbs of horses, where it continuously grows rapidly and may reach vast quantities,[4-8] we hypothesized that the growth rates of these granulation tissue–derived cells may have been much higher than the growth rates of the normal cells. However, in vivo the granulation tissue is a highly vascular, resistant tissue which suggests that the slower growth rates in vitro may be due to lack of naturally occurring nutrients or growth factors which may be present in the granulation tissue.

All three cell types had distinctive morphologic changes with the addition of TGF-β to the medium, which may be due to unnaturally high concentrations of these growth factors. However, a more likely explanation is that these cells need to have contact with other extracellular matrix molecules, that is, collagen or fibronectin.

In the growth rate studies TGF-β inhibited the proliferation of the normal and sarcoid-derived cells but failed to inhibit growth of the granulation tissue cells, as did other growth factors. TGF-β can increase the rate of cell proliferation in some fibroblast cell types by means of induction of a platelet-derived growth factor–like protein,[21] but its predominant effect is to inhibit cell growth.[22] It is probable that the addition of TGF-β neutralizing antibody would reverse the effect that TGF-β had on the granulation tissue–derived cells. Shah et al.[23] found the application of TGF-β neutralizing antibody at the time of wounding was essential to reduce active TGF-β levels, limit macrophage infiltration, and further TGF-β release. Higher levels of endogenous TGF-β may be present in the granulation tissue than in the other tissues used for primary culture.

EGF was strongly stimulatory for normal cells with cell numbers steadily increasing over time. The same effect was seen with sarcoid cells but to a lesser extent, which could be due to sarcoid cells having fewer EGF receptors on their cell surfaces than normal cells. Normal human cells have approximately 20,000 EGF receptors per cell.[24] Increased proliferation is a common cellular response to EGF,[25,26] but EGF had an inhibitory effect on the granulation tissue–derived cells. The sarcoid- and granulation tissue–derived cells also showed altered morphology in the presence of EGF. Cohen, Carpenter, and Lembach[26] reported EGF to cause distinct morphologic alterations to cells.

FGFb was biologically more potent for the sarcoid-derived cells than FGFa. Fibroblast growth factors stimulate proliferation of most major cell types involved in wound healing both in vivo and in vitro.[27] In addition, FGFb induces cell migration, neovascularization, and the formation of granulation tissue in animal models. In our studies we found both FGFa and FGFb to be inhibitory for the granulation tissue proliferation. However, in the in vivo situation it could be that the granulation tissue fibroblasts within the tissue are producing excess FGFb which is in turn stimulating exuberant granulation tissue formation. This excess production of FGFb could be due to the fact that these cells have a larger number of receptors for FGFb or they may be influencing the secretion of other growth factors. It may therefore be useful to measure quantities of TGF-β and FGFb in the conditioned medium from these cells to establish whether the levels are elevated above normal. Isolation and characterization of these factors and others which may be present in the conditioned medium may show the presence of novel growth factors. Identification of these factors and the mechanisms that regulate their expression and activity will be important in understanding the processes of equine wound healing.

ACKNOWLEDGMENTS

This work was supported by the Home of Rest for Horses, for which the authors are immensely grateful. We thank Professor Rudland, University of Liverpool, for his donation of FGFb.

REFERENCES

1. Silver IA. Basic physiology of wound healing in the horse. Equine Vet J 1982;14:7-15.
2. Bertone AL. Principles of wound healing. Vet Clin North Am Equine Pract 1989;5:449-63.
3. Freeman KL, Knottenbelt DC, Cochrane CA. Growth characteristics of some cells involved in equine wound healing. Res Vet Sci In press.
4. Jacobs KA, Leach DH, Fretz PB, Townsend HGG. Comparative aspects of the healing of excisional wounds on the leg and body of horses. Vet Surg 1984;13:83-90.
5. Bertone AL. Control of granulation tissue on equine limbs: a clinical update. Vet Rep 1988;1:6-9.
6. Bertone AL, Sullins KE, Stashak TS. Effect of wound location

and the use of topical collagen gel on exuberant granulation tissue formation and wound healing in the horse and pony. Am J Vet Res 1985;46:1438-44.

7. Fretz PB, Martin GS, Jacobs KA. Treatment of exuberant granulation tissue in the horse. Evaluation of four methods. Vet Surg 1983;12:137-40.
8. Lindsay WA. Wound treatment in horses: what to know about second-intension healing. Equine Pract 1988;10:396-403.
9. Jackson C. The incidence and pathology of tumors of domestic animals in South Africa. A study of the Onderstepoort collections of neoplasms with special reference to their histopathology. Onderstepoort J Vet Res 1936;6:1-460.
10. Head KW. Some data concerning the distribution of skin tumors in domestic animals. In: Rook AJ, Walton GS, editors. Comparative physiology and pathology of the skin. Philadelphia: F.A. Davis Co., 1965: 615-36.
11. Knottenbelt DC, Pascoe RR. Color atlas of diseases and disorders of the horse. Prescott: Wolfe Publishing, 1994: 260-1, 275-9.
12. Knottenbelt DC, Walker JA. Topical treatment of the equine sarcoid. Equine Vet Educ 1994;6:72-5.
13. Ragland WL, Keown GH, Spencer GR. Equine sarcoid. Equine Vet J 1970;2:2-11.
14. Williams IF, Heaton A, McCullagh KG. Connective tissue composition of the equine sarcoid. Equine Vet J 1982;14:305-10.
15. Genetzky RM, Biwer RD, Myers RK. Equine sarcoid. Causes, diagnosis and treatment. Compend Cont Educ Pract Vet 1983;5:416-20.
16. Tarwid JN, Fretz PB, Clark EG. Equine sarcoids: a study with emphasis on pathologic diagnosis. Compend Cont Educ Pract Vet 1985;7:293-300.
17. Adams R, Calderwood-Mays MB, Peyton LC. Excision of cutaneous tumors in the horse using histologic guidance. Vet Surg 1988;17:241-5.
18. Olsen C Jr, Cook RH. Cutaneous sarcoma-like lesions of the horse caused by the agent of bovine papilloma. Proc Soc Exp Biol Med 1951;77:281-4.
19. Ragland WL, Spencer GR. Attempts to relate bovine papilloma virus to the cause of equine sarcoid. Immunity to bovine papilloma virus. Am J Vet Res 1968;29:1363-6.
20. Ragland WL, Spencer GR. Attempts to relate bovine papilloma virus to the cause of equine sarcoid, equidae inoculated intradermally with bovine papilloma virus. Am J Vet Res 1969;30 743-51.
21. Leof H, Proper JA, Goustin AS, Shipley GD, Dicorleto PE, Moses HL. Induction of c-sis mRNA and activity to platelet-derived growth factor–like activity by transforming growth factor type B: A proposed model for indirect mitogenesis involving autocrine activity. Proc Natl Acad Sci U S A 1986;83:2453-7.
22. Moses HL, Tucker RF, Leof EB, Coffey RJ, Halper J, Shipley GD. Type B transforming growth factor is a growth stimulator and a growth inhibitor. Cancer Cells 1985;3:65-71.
23. Shah M, Foreman DM, Ferguson MWJ. Control of scarring in adult wounds by neutralising antibody to TGF-β. Lancet 1992;1:213-4.
24. Gill GN, Bertics PJ, Santon JB. Epidermal growth factor and its receptor. Mol Cell Endocrinol 1987;51:169-86.
25. Armelin HA. Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc Natl Acad Sci U S A 1973;70:2702-6.
26. Cohen S, Carpenter G, Lembach KJ. Interaction of epidermal growth factor (EGF) with cultured fibroblasts. Adv Metab Disord 1975;8:265-84.
27. Cordon-Cordo C, Vlodavsky I, Haimovitz-Friedman A, Hicklin D, Fuks Z. Expression of basic FGF in normal human tissue Lab Invest 1990;63:832-40.