THE ROLE OF MACROPHAGES IN HUMAN WOUND HEALING AND THEIR RESPONSE TO A TISSUE ENGINEERED DERMAL REPLACEMENT IN HUMAN CHRONIC WOUNDS

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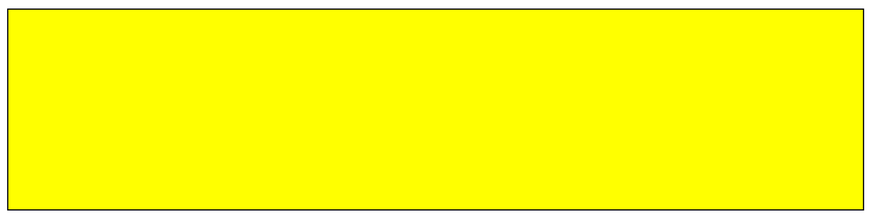
STATEMENTS

STATEMENT 1

With the exception of preparing and analysing the clinical data and the wax sections for the Dermagraft study performed by the staff at Smith and Nephew, York, UK, all practical work submitted in this thesis is entirely the result of the candidates own investigation.

Signed: 

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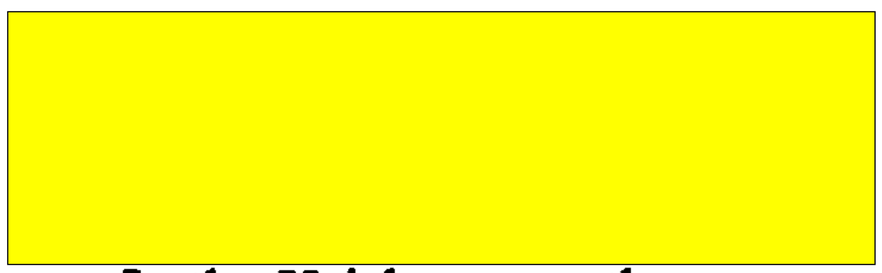
Signed: 

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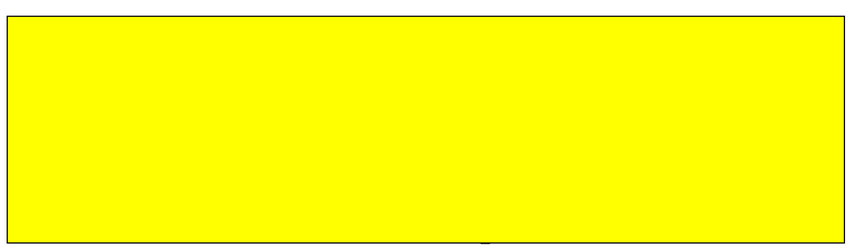
Signed: 

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LIST OF ABBREVIATIONS

AE	Adverse Event
b-FGF	Basic Fibroblast Growth Factor
Ca^{2+}	Calcium
CEA	Cultured Epidermal Autograft
CMV	Cytomegalovirus
CVA	Cerebro-vascular Accident
DAB	Diaminobenzidine
DG	Dermagraft
DNA	Deoxyribo Nucleic Acid
ECM	Extra Cellular Matrix
ED_{50}	Effective Dose 50
EGF	Epidermal Growth Factor
GM-CSF	Granulocyte – Macrophage Colony Stimulating Factor
GP	General Practioner
H&E	Haematoxylin and Eosin
HA	Hyaluronic Acid
HIV	Human Immunodeficiency Virus
Hpf	High Power Field
HSV	Herpes Simplex Virus
HTLV	Human T-cell Lymphotrophic Virus
ICAM	Intercellular Adhesion Molecule
IEC	Independent Ethics Committee
IFN	Interferon

IL	Interleukin
IRB	Institutional Review Body
kDa	Kilo Dalton
LPS	Bacterial Lipopolysaccharide
LREC	Local Research Ethics Committee
mmHg	millimetres of Mercury
MMP	Matrix Metalloproteinase
MR	Mannose Receptor
mRNA	Messenger Ribo Nucleic Acid
MRP	Migration inhibition factor Related Protein
NK	Natural Killer Cells
PDGF	Platelet Derived Growth Factor
PNS	Pilonidal Sinus
SR	Sialoadhesin Receptor
TBS	Tris Bufferd Saline
TGFα	Transforming Growth Factor Alpha
TGFβ	Transforming Growth Factor Beta
TIMP	Tissue Inhibitors of Metalloproteinases
TNFα	Tumour Necrosis Factor Alpha
VCAM	Vascular Cell Adhesion Molecule
VEGF	Vascular Endothelial Growth Factor
VLU	Venous leg Ulcer
W/D	Withdrawn

SUMMARY

The vast majority of current researches involving wound healing processes have been undertaken utilising animal models with minimum involvement with human subjects. The major cellular component of healing after neutrophils, are the macrophages.

This thesis examines the role of macrophages in wound healing, concentrating on the differences in the subpopulation of wound macrophages in acute and chronic wounds. Examining 20 human wound bed biopsies (pilonidal sinus and venous leg ulcers), significant differences were observed between the subpopulations of wound macrophages in acute healing and chronic non-healing wounds. The results exhibit that within the acute wound there is an accumulation of early stage macrophages (mean 20.8, SD 8.6) differentiating from monocytes which become activated and contribute to the wound healing process. There being few early stage macrophages within the chronic wound (mean 10.4, SD 6.7) ($p \leq 0.01$). Chronic wounds in comparison demonstrated a significant accumulation of tissue macrophages (mean 34.0, SD 10.5) when compared to acute wounds (mean 10.9, SD 4.4) with limited wound healing ($p \leq 0.01$).

The dermagraft (DG) study comprising of 53 patients, showed that applying a biologically active dressing (1-12 dressings over 12 weeks) and compression to the wound bed, exhibited complete closure (76%) or reduction in the size of the wounds at 12 weeks, compared to compression alone. Changes in the extracelluar components and an array of inflammatory cells and cytokines in fifty three paired wound bed biopsies (106) with and without DG were examined at week 0 and week 6 of treatment.

On histological investigation, DG exhibited an increase in the amount of collagen present and angiogenesis in the wound at week 6 of treatment.

Although there were no significant changes in the lymphocyte counts in response to the application of DG, it was possible to demonstrate a significant increase in the number of early stage macrophages at week 6 of treatment ($p \leq 0.05$) and a significant reduction in the tissue macrophage counts, at week 6 of treatment ($p \leq 0.05$) in patients treated with 4 pieces of DG.

The levels of different cytokine expression within the wound bed at week 6 exhibited some changes but this was not significant, in response to DG treatment. This could be to the possible presence of proteinases within the chronic wound bed hydrolysing the cytokines produced by DG.

From the results attained, it was able to conclude, for clinical use 4 pieces of DG at regular dosing intervals were sufficient to achieve wound contraction or closure. This dose regimen has now been taken forward for further pivotal studies.

This thesis thus represents some of the first evidence in human tissue that macrophages may play a role in wound healing, and in chronic wounds, a subpopulation of macrophages can be modified to stimulate these wounds towards healing.

1 CHAPTER ONE: INTRODUCTION

1.1 HISTORY OF WOUND HEALING

In the evolutionary process, humans traded the slow biological sophistication of regeneration, seen in many less complex organisms, for the relatively simple and more rapid healing of wounds. The oldest manuscripts known to man that record the care and treatment of wounds dates back to around 2200 BC, in the form of clay tablets. These tablets describe the three healing gestures of – *Washing the wound, Making plasters, and Bandaging the wound.*

Throughout the ages, a variety of different methods and substances have been used to treat wounds. Recipes sometimes consisted of dozens of different ingredients, mixed in a precise manner, and often requiring the assistance of a priest, magician or apothecary.

One of the earliest known wound care products was beer!! Wound dressings, often referred to as plasters were designed to provide protection to the healing wound and to absorb exudates. These were made from different substances, including mud or clay, plants and herbs. In addition, the most common ingredient used in plasters was oil.

Ancient Egyptians were the first to document wound management treatments. (Sipos P et al.2004) From the Smith and Ebers papyri, dating back to 1650 BC and 1550 BC, respectively, it can be shown that they were the first people to prepare plasters and apply bandages, including the application of more ingenious dressings such as 'goat dung soaked in yeast that is fermenting', or 'a frog warmed in oil'! However, honey, grease, resins and lint were the more common components of Egyptian plasters (Majno G, 1975).

The Egyptians were also known to use meat to cover wounds, similar to the way pig skin, or allografts are used today. They were also the first to distinguish between what

they called 'bad' or 'infected' wounds, and 'good' or 'uninfected' wounds. The production of pus was considered beneficial to healing 'sick' wounds, although the scientific basis for the concept of infection would not emerge for several millennia. They left an immense influence on the Greeks and Romans, and hence the Western world.

The Greeks proceeded to improve on the knowledge of the Egyptians. Hippocrates (460 – 375BC) was the first to cleanse the wounds with wine and vinegar. Wounds were treated with applications of figs and brambles in an attempt to alleviate the symptoms of inflammation, haemostasis or gangrene. The Greeks also understood the value of pressure relief at the ulcer site and usually recommended that the patient stayed off their feet (Forrest RD, 1982). Hippocratic physicians believed that all diseases including wounds could be cured by restoring the humoral balance or eliminating the 'bad humor' by blood letting. Blood letting in an attempt to heal wounds was practised extensively, as the physicians believed that even if a person died during blood letting, he died 'healthy'. They also developed the technique of ligation of blood vessels and hence became more adventurous in blood letting.

The cardinal features of inflammation: calor (heat), rubor (redness), tumour (swelling), and dolor (pain), was not described until Roman times by Celsus. (Rutkow IM, 1993, Naylor IL, 1999).

With the fall of Rome, the Arabs further perfected the art of blood letting, but it was felt that as the sick were unclean, blood letting should be performed by barbers or surgeons under the supervision of physicians.

Wound care in the Middle Ages actually regressed with the loss of most of the Greek and Roman writings.

Theodoric of Bologna (1205 – 1296), who trained at the University of Bologna, believed that the production of pus in a wound was actually deterimental to healing and promoted a standardized method of wound healing which included cleaning the wound with acetic acid, removing dead tissue by debridement, closing the wound with stitches, and applying a bandage. Unfortunately this practise was abandoned after the death of his pupil, the French surgeon Henri de Mondeville. This was mainly due to the accusations of plagiarism by another famous French surgeon, Guy de Chauliac (Popp AJ, 1995), and was abandoned for 700 years.

The next milestone in wound healing occurred during the time of Ambroise Paré (1510 – 1590). He was an army surgeon during the reign of King Henry II in 1552. He developed a standard method of managing gunshot wounds which involved treating them with hot oil and cauterisation. During a particularly bloody battle in 1536, Paré ran out of hot oil and was forced to find and alternative. He used a mixture of turpentine, rose oil and egg whites to treat the wounds of soldiers. In his own words he wrote " *....I found such as I had dressed with the digestive only, free from vehemence of pain to have good rest, and that their wounds were not inflamed, ... but on the contrary the others that were burnt with scalding oil were feverish, tormented with much pain, and the parts about their wounds were swollen*"

From then on, he vowed never to treat injuries with hot oil.

Cle de Villars in 1740, noted that wounds left open to the environment healed less effectively than wounds that were closed. This resulted in the birth of occlusive dressings. John Hunter (1728 – 1793), the 'Father of Modern Surgery', was one of the dominant personalities in medicine. He noted that wounds were able to heal under a scab when left undressed (Ellis H. 2001). He was also the first surgeon to advocate the laying open of narrow wounds and sinus tracts, which subsequently healed by

secondary intention. He distinguished 'adhesive' inflammation which was amenable to surgical intervention, from 'suppurative' inflammation, which delayed healing, demonstrating this was related to infection (Leaper DJ, 1998).

In the nineteenth century major advances in wound healing were developed. The cause of infection and its prevention was recognised. Progress was made towards the greater understanding of the cellular processes that contributed to the inflammatory course of wound healing. Joseph Lister (1827 – 1912) realised that bacteria were the cause of wound sepsis. However he realised that Louis Pasteur's (1822 – 1895) method of destroying bacteria by heating could not be applied to living tissue. In his search to find an antiseptic he tried many chemicals and discovered that carbolic acid or phenol were most effective (Toledo – Pereyra LH and Toledo MM. 1976). The results were dramatic, and within a few years, the antiseptic techniques of soaking the surgical instruments, sutures and lint dressings, and spraying the operating theatre with carbolic spray became standard procedure. Other pioneers in the evolution of asepsis were Ernst von Berman (1836 – 1907), who developed the method of steam sterilisation of instruments, and William Stewart Halsted (1852 – 1922) who actively promoted the use of rubber gloves in surgery to improve wound hygiene (Rutkow IM, 1993).

Understanding the biology of wound healing began with Virchow when he published a book in 1860 – '*Cellular pathology as based on physiological and pathological history*' and Elie Metchnikoff's '*The identification of phagocytic cells in the process of wound healing*', in 1890. With the invention of the electron microscope in 1930, the cellular and humoral immunology of wound healing grew rapidly.

The first researcher to study the biology of wound healing in humans was Shattuck W Hartwell in 1926, who presented his thesis, entitled '*The mechanism of healing in*

human wounds' to the University of Minnesota (Butterworth RJ, 1992,). Hartwell suggested that human wound healing differed from studies performed on other animal species.

He looked at 850 sections from a variety of wounds in experimental animals such as rabbits, dogs, pigs, pigeons and human.

He found that although epithelialisation was similar in the human and animal wounds, the fibrous healing differed consistently in several ways:

- There were more mitoses apparent in the animal wounds
- In the human, fat was considered the main locus of healing, but the pattern of subcutaneous fat in animals was different
- Animal wounds had larger vessels than human
- The morphology of cells identified by Hartwell as macrophages was different

He thus concluded that '*the conception of healing in human tissue should rightly be built up from observations of human tissues. The fact that the histological findings are different in animals from those in human wounds merely emphasizes this fact. The differences serve to explain why the description of wound healing in human surgical wound does not correspond more closely to the descriptions of healing given in text books which are based on experimental wounds in animals.* (Hartwell SW 1930, Hartwell SW. 1955).

The last few years have seen a number of interesting discoveries in the understanding of the healing process, mostly based on animal wound models. From being merely scavenger cells to 'mop up' debris in wounded tissue, macrophages have been shown

to play an integral role in the process of wound healing, ranging from wound debridement to matrix formation (Leibovitch SJ and Ross R, 1975). Efforts have also been made to stimulate healing in recalcitrant wounds by the application of topical growth factors such as Platelet Derived Growth Factor (PDGF) (Pierce GF et al, 1995, Krupski WC et al, 1991), autografts in the form of a split thickness skin graft, and the use of tissue engineered products (Schonfield WH et al, 2000, Leigh IM and Purkis PE, 1986).

The future of wound healing no doubt holds exciting new discoveries.

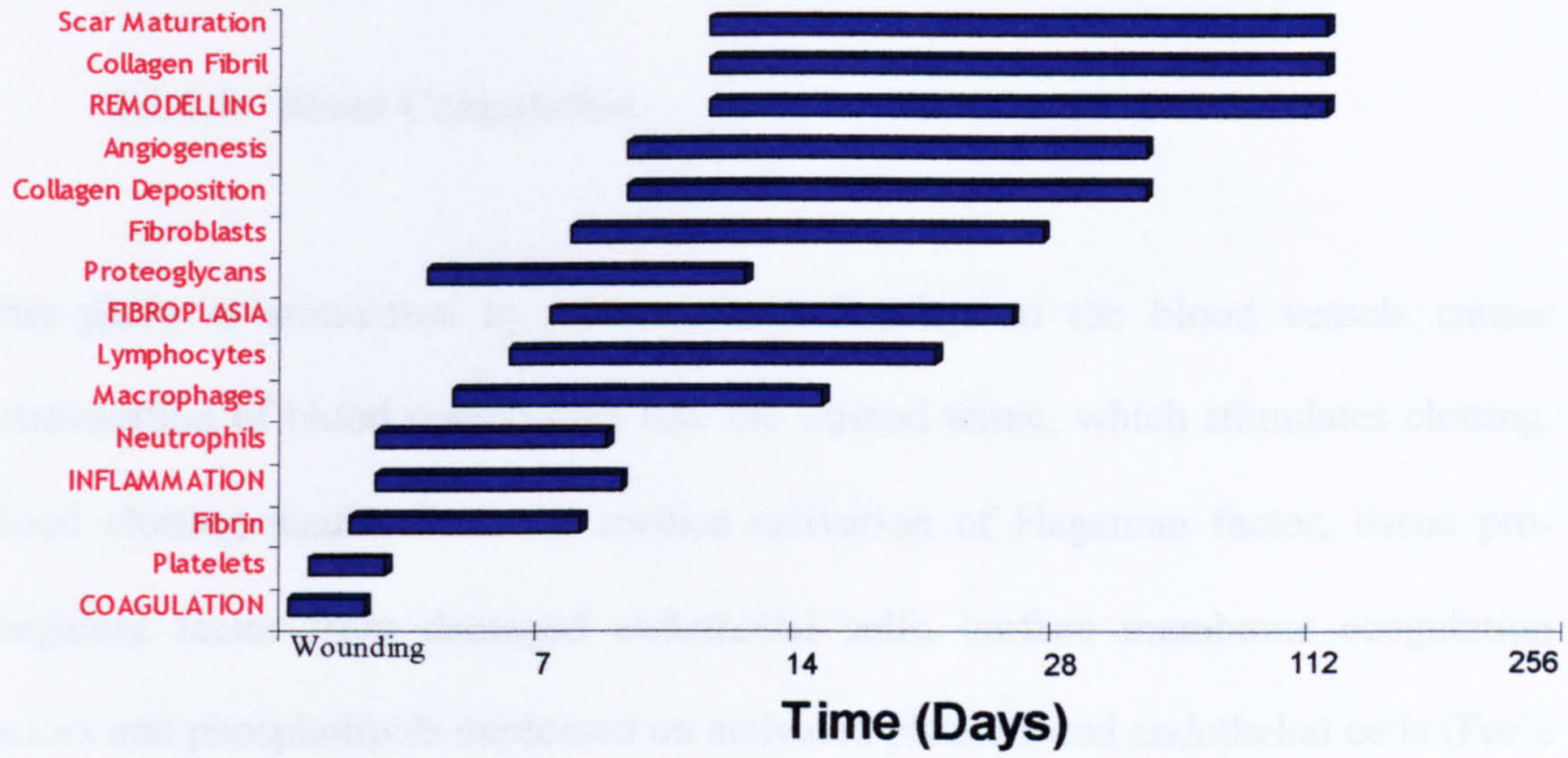
1.2 OVERVIEW OF WOUND HEALING

Cutaneous injury can take many forms – surgical trauma, burns, and immunologically mediated injury are just a few in a long list. Despite this, the general sequence of events that are activated in response to injury and subsequent wound repair show striking similarity irrespective of the initial insult. Cutaneous wound healing proceeds as a fibroproliferative response that develops into a fibrotic scar. Thus the organ is patched rather than restored. Wound repair is not a simple linear process in which growth factors released by physiologistic events activate inflammation, parenchymal cell proliferation and migration, but rather it is an integration of dynamic interactive processes involving soluble mediators, formed blood elements, extracellular matrix and parenchymal cells. Alterations in the normal healing processes produce less desirable outcomes. A plethora of pathobiological states such as diabetes (Falanga V. 2005), venous hypertension (Trent JT et al. 2005), poor arterial perfusion (Mogford JE et al.2003), or persistent infection (Singer AJ and McLain SA. 2002) can lead to non-healing wounds or excessive scarring

In humans, apart from in the foetus, this sequence of events eventually leads to the formation of scar tissue as a result of abundant collagen synthesis by fibroblasts that proliferate and differentiate within the provisional matrix. Only hepatic and epithelial cells (in superficial damage) are capable of complete regeneration.

The wound repair processes follow a specific time sequence and can be temporarily categorised into three major events: Inflammation, Tissue formation, and Tissue remodelling (Howes EZ et al., 1929). The three phases are not mutually exclusive, but overlap in the time sequence of wound healing. (Figure 1-1)

Figure 1-1 Progression of Wound Healing – key events



Adapted from Mast 1992 (Mast BA, 1992)

In chronic non-healing wounds encountered in patients with venous leg ulcers, this sequence of events is arrested in one or more stages of this progression with the development of a 'progression-regression' pattern of healing. Although there is significant knowledge of the processes involved in normal healing, the disordered processes involved in the biology of abnormal wounds are more difficult to explain.

1.2.1 The Inflammatory Phase

This phase commences within minutes of tissue injury resulting in the arrest of bleeding, clearing the wounded tissue of bacteria, removal of non-viable tissue or debris, and the formation of a fibrin rich clot. The clot has a dual function of plugging

severed vessels and acting as a scaffold for cells, thus preparing the wounded tissue for the next phase.

1.2.1.1 Blood Coagulation

This phase is stimulated by tissue injury. Damage to the blood vessels causes extravasation of blood constituents into the injured tissue, which stimulates clotting. Blood clotting results from the surface activation of Hageman factor, tissue pro-coagulant factor from damaged endothelial cells, surface membrane coagulation factors and phospholipids expressed on activated platelets and endothelial cells (Furie B and Furie BC, 1988).

The critical event in all cases is the availability of a surface of damaged blood vessel wall, with its exposed collagen, to promote adsorption and activation of coagulation pro-enzymes such as fibinogen and prothrombin. Minute spontaneous activation of these proenzymes also contribute to the positive feedback loop and results in the amplification of the physiological response of coagulation.

The coagulation cascade ceases with the production of prostacyclin, which inhibits platelet aggregation, antithombin III binding to thrombin and thus inhibiting its activity, protein C a potent enzyme that degrades coagulation factors V and VIII, and the release of plasminogen activator which initiates clot lysis by converting plasminogen to plasmin (Moncada S et al., 1976, Stern DM et al., 1985, Loedam JA et al., 1988, Loskutoff DJ and Edgington TS, 1977).

Haemostasis is the main function of blood coagulation, but the clot, which is rich in fibrin, also provides a matrix scaffold for the recruitment of cells to the injured area.

Specifically the fibrin acts as a provisional matrix for the influx of monocytes (Lanir N et al., 1988) and fibroblasts (Brown LF et al., 1993b)

1.2.1.2 Platelets

These are found in the intravascular space in an inert form. Haemostasis is dependent on platelet adhesion and aggregation. The platelets adhere to the damaged interstitial connective tissue and then aggregate. During aggregation, they become activated secreting multiple mediators, including fibrinogen, fibronectin, thrombospondin and von Willebrand factor VIII, amongst others. Fibrinogen, fibronectin, and thrombospondin act as ligands for platelet aggregration and platelet fibrinogen is also converted to insoluble fibrin which adds to the fibrin clot. (Ginsberg MH et al., 1988). Platelets also release chemotactic factors for blood leucocytes, (Weksler BB, 1992) growth factors such as platelet-derived growth factor (PDGF), (Ross RR and Raines EW, 1990) and transforming growth factor-alpha and beta (TGFα and β) (Dernyk R, 1988, Sporn MB and Roberts AM, 1992) which regulates the formation of new tissue.

1.2.1.3 Neutrophils

Neutrophils and monocytes enter the wounded tissue at the same time (usually within hours after injury), but because of the greater number of neutrophils in the general circulation, they tend to be the predominant cell type in the early phase of inflammation. They are chemotactically attracted to the injured tissue by fibrin degradation products, such as fibrinopeptides cleaved from fibringen, C5a from the activated classical or alternative complement cascades, leukotriene B_4 released from activated neutrophils, formyl methionyl peptides from bacterial proteins and PDGF from platelets (Williams TJ, 1988).

Activated neutrophils also release elastase and collagenase, which facilitate cell penetration through vessel basement membranes (Tonnesen MG et al, 1988). At the wound site they destroy contaminating bacteria by phagocytosis and subsequent enzymatic and oxygen radical mechanism (Klebanoff SJ, 1992). If wound contamination is minimal, neutrophil infiltration ceases within a few days. Early stage macrophages are also armed with collagenases which facilitate the debridement of non - viable tissue (Campbell EJ et al., 1987).

1.2.1.4 Monocytes

The accumulation of monocytes at the site of injury persists by selective monocyte chemoattractants. These include fragments of collagen (Postlethwaite AE and Kang AH, 1976), elastin (Senior RM et al., 1980), fibronectin (Clark RAF, 1988), enzymatically active thrombin (Bar-Shavit R et al., 1983), and TGFβ (Wahl SM et al.,

1987). A detailed review of the role of monocytes and macrophages will be discussed in later chapters.

1.2.2 The Proliferative Phase

This phase commences at about the second day of injury and lasts about four weeks in normal healing wounds. During this phase, there is a marked influx of macrophages and fibroblasts into the wound along the fibrin scaffold laying down a variety of substances including hyaluronic acid, chronditin-4-sulphate, dermatan sulphate, heparan sulphate constituting the amorphous ground substance within the wound. There is also an increased synthesis of collagen. This increases the tensile strength of the wound.

1.2.2.1 Granulation Tissue

During the early phase of cutaneous wound repair, new stroma, often called granulation tissue, begins to form approximately four to five days after injury. Granulation tissue consists of a dense population of inflammatory cells, fibroblasts and new blood vessels embedded in a loose matrix of collagen, fibronectin and hyaluronic acid. The large number of new capillary loops on the surface of the granulation tissue creates a granular appearance, hence its name.

This is sometimes referred to as the proliferative phase of wound healing. In this phase the formation of new blood vessels –*Angiogenesis*, and the laying down of the extracellular matrix and collagen– *fibroplasia* are the major events. The granulation tissue restores the soft tissue defect by creating new connective tissue and blood

vessels, which forms a scaffolding for the migration of epithelial cells (Martin P, 1997, Witte M and Barbul A, 1997).

In healthy wounds granulation tissue appears as a firm, pink tissue. It is the prerequisite to re-epithelialisation. O*ver*granulation observed in patients with chronic wounds is thought to be due to defective signaling or a result of infection, thus preventing re-epithelialisation.

1.2.2.2 Angiogenesis

This term was first used by Hertig in 1935 to describe new blood vessel formation in the placenta, and it has been extensively studied in chick chorioallantoic membrane and the cornea by Folkman and Shing (Folkman J and Shing T, 1992). Angiogenesis in the healing wound usually starts at the same time as fibroplasia and is an essential part of wound healing. A number of experimental systems, such as the rabbit ear chamber, corneal and chorioallantoic models (Folkman J and Klagsburn M, 1987), have been used to study the cellular sequence of events in the process of neovascularisation, but the regulators of these processes are still poorly understood. The process begins with the disruption of the basement membrane of the parent vessel by specific proteases probably released by endothelial cells. These proteases also breakdown the adjacent extra cellular matrix (Rifkin BD et al., 1982, Magnatti P et al., 1989). This creates tissue defects that facilitate the formation of capillary sprouts that form the basis of capillary plexuses (Folkman J, 1982).

Cellular proliferation occurs in the endothelial cells remaining within the parent vessel, which then migrate in continuity with the lead cells. The capillary sprouts link up to form loops through which blood begins to flow. The process of budding repeats

itself from the new vessels forming a plexus, supplying nutrients and oxygen to the wounded tissue.

Angiogenesis is regulated by a complex interplay between the hypoxic environment, and growth factors such as basic fibroblast growth factor (bFGF) platelet derived growth factor (PDGF), vascular endothelial growth factor (VEGF). (Tonnesen MG et al., 2000)

1.2.2.3 Fibroplasia – matrix deposition and collagen synthesis

Clot formation, during the inflammatory phase provides the initial fibrin matrix to support cell migration. Under the influence of platelets and macrophages, secreting PDGF and TGFβ in the injured tissue, fibroblasts enter the wound environment and undergo a series of changes (Seppa HEJ et al., 1982, Postlewaite AE et al., 1987). Moving into a cross-linked fibrin blood clot needs the activity of a proteolytic system that can cleave a path for the migration. A variety of fibroblast-derived enzymes and serum derived plasmin, including plasminogen activator, interstitial collagenases [Matrix metalloproteinase 1(MMP-1)], gelatinises [MMP-2] and stromelysin [MMP-3] aid the migrating fibroblasts (Grant GA et al., 1987, Saus J et al., 1988, Stetler-Stevenson WG et al., 1989). The endoplasmic reticulum and golgi apparatus retract to a perinuclear position within the fibroblast. This is followed by the formation of loose extra cellular matrix composed of fibronectin. (Grinnell F et al., 1981).

Under the influence of TGF-β the fibroblasts change their phenotypic composition and increase collagen production (Ignotz RA and Massague J, 1986). Once abundant collagen matrix is deposited into the wound, fibroblasts cease collagen production despite the continuing expression of TGF-β. Grinnell in 1994 has demonstrated that

the collagen matrix itself can suppress both fibroblast proliferation and collagen production (Grinnell F, 1994). It has been found that many fibroblasts in a 10-day healing wound develop pyknotic nuclei, thus resulting in apoptosis (programmed cell death), but the triggers for wound fibroblast apoptosis has not been elucidated (Williams GT, 1991). This converts a fibroblast-rich granulation tissue to a relatively acellular scar. From this one can appreciate that fibroplasia in wound repair is tightly regulated and when this regulation is disrupted, excessive scarring may occur such as in keloid formation and scleroderma.

1.2.2.4 Epithelialisation

Rapid re-establishment of any epithelial barrier decreases significant morbidity. One or two days after injury, epithelial cells at the wound margin begin to proliferate. The epithelial cells undergo marked phenotypic changes, which include retraction of intra-cellular tonofilaments, dissolution of most inter-cellular desmosomes and the formation of peripheral cytoplasmic actin filaments which aid their mobility (Gabbiani G et al., 1978). Desmosomes are structures that interlink epithelial cells thus providing strength to the epithelium. The binding between the epidermis and the dermis is also lost. This allows wound epidermal cells to extend pseudopodia from their free basolateral edge and move into the wound. Migrating wound epidermal cells do not contain the keratin proteins that are found in mature stratified epidermis or the matrix protein flaggrin, in which the keratin are embedded. Cells in all layers of the migrating tip contain the type of keratin that is normally found in the basal layer of the stratified epidermis (Mansbridge JN and Knapp AM, 1987).

The stimulus for these changes are unknown, however it has been postulated that the lack of neighbouring cell contact may be the stimulus for both epithelial migration and proliferation, similar to the way re-endothelialisation occurs in large blood vessels after intimal damage (Heimark RL and Schwartz SM, 1988). Growth factors such as epidermal growth factor family [EGF], transforming growth factor α [TGFα], and fibroblast growth factor family [FGF] may also play a role in the migration of keratinocytes (Clark RAF, 1996).

Migrating keratinocytes dissect through the wound, breaking down slough and debris by secreting collagenases and plasminogen activators (Woodley DT et al., 1986, Grondahl-Hansen J et al., 1988).

Once keratinocytes have migrated over the provisional matrix, there is re-establishment of the elements of the basement membrane. These include collagen type IV, heparan sulphate and the attachment factor laminin. The components of epidermal intracellular junctions, desmosomes and hemi-desmosomes are assembled, and the anchoring fibrils are replaced. The epidermis then enters a regenerative phase in which a mature stratum corneum is produced and is anchored to the underlying neodermis through type VII collagen (Gipson IK et al., 1988).

If the wound is superficial, there is preservation of epidermal appendages such as eccrine sweat glands, sebaceous glands, and hair follicles, these become the major source of epidermal cells, a principle utilised in the healing of donor sites after harvesting a split thickness skin graft (Glat PM and Longaker MT, 1997).

1.2.3 The Maturation Phase

1.2.3.1 Tissue remodelling

The final phase of wound healing is an ill-defined overlapping phase that begins at the time of matrix deposition in the wound and persists for many years after wound closure by the epithelium. By observing the strength of dermal scars in animal models with time from injury, it can be seen that the initial deposition of collagen fibres are random, but soon align perpendicular to the wound providing strength to the scar tissue (Ehrlich HP., 1998). There is also a change in the composition of the collagen bundles as the scar tissue matures. Early collagen, known as type III collagen, makes up 30% of the granulation tissue, and contributes little to the wound's tensile strength (Kurkinen M et al., 1980). As the collagen matures, type III collagen is replaced by type I collagen. Therefore there exists a simultaneous process of matrix breakdown and synthesis. Fibroblasts are responsible in the regulation of these dual processes, by producing the extracellular components and MMPs, which are responsible for the degradation of the matrix (Mast BA and Shultz G., 1996). During this process the scar gains tensile strength, but only approaches 80% of the tensile strength of unwounded tissue. (Lindblad WJ, 1998)

Imbalance in matrix synthesis and breakdown results in abnormal wound healing.

This is seen in hypertrophic scars, keloid formation, and non healing chronic wounds.

1.3 THE EXTRACELLULAR MATRIX IN WOUND HEALING

In acute wounds granulation tissue forms in an orderly sequence, initially this consists of fibronectin, hyaluronic acid, then collagen type III and finally collagen type I as highlighted in the previous section.

1.3.1 Fibronectin

Fibronectin is a multidomain, multifunctional cell adhesion protein found in blood and in a variety of tissue extracellular matrices (Hynes RO, 1990). It fibrillar glycoprotein of 440kDa and is a key component of the provisional matrix in the early stages of wound repair. Fibroblasts, macrophages, endothelial cells and keratinocytes are known to secrete fibronectin (Colvin RB et al., 1979, Brown LF et al., 1993a, Kubo M et al., 1984)

Fibronectin is present in the initial clot with fibrin and acts as a stimulus for its own production by fibroblasts, macrophages, endothelial cells and keratinocytes, via a positive feedback system. It has several roles during the wound healing process. It acts as a chemotactic factor for peripheral blood monocytes, as a non-specific opsonic enhancer, is chemotactic for cell movement, provides a substratum for cell attachment and new matrix deposition (Brotchie H and Wakefield D, 1990).

Monocytes are attracted to fibronectin or proteolytic fragments of fibronectin containing 'cell binding' domains (Norris DA et al., 1982, Akiyama SK et al., 1994). This results in an accumulation of monocytes at the site of wounding (Senior RM et al., 1980).

Fibronectin and it's fragments also stimulate fibroblast and endothelial cell movement, but at present it is unclear whether this movement is secondary to chemotaxis (cells migrating in response to a fluid phase chemical gradient) or haptotaxis (cells migrating in response to a semi-solid surface bound chemical gradient (Postlethwaite AE et al., 1981, Bowersox JC and Sorgente N, 1982).

Tissue debris, such denatured collagen in wounds when coated with fibronectin appears to be ingested by macrophages (Martin DE et al., 1988, Van de Water III L et al., 1981). Here fibronectin functions as a opsoniser for more effective clearance of small particles from the wounded tissue by macrophages, fibroblasts and epidermal cells (Gudewicz PW et al., 1980, Grinnell F et al., 1981, Takahima A and Grinnell F, 1985).

Fibronectin provides a scaffold for the assembly of collagen matrix. This is evidenced by the fact that its appearance in the wound precedes collagen deposition, and preventing or disrupting the fibronectin matrix results in the disruption of the collagen matrix (Welch MP et al., 1990, Keski Oja T et al., 1981).

1.3.2 Hyaluronic Acid (HA)

Hyaluronic acid is a linear polysaccharide consisting of alternating units of N-acetyl glucosamine and D-glucouronic acid, giving a molecular weight of between 100 and 5000 kDa (Manuskiatti W and Maibach HI, 1996). HA is synthesised at the cell membrane and is particularly abundant around fibroblasts and other mesenchymal cells, where HA chain elongation occurs by membrane linked enzyme, HA synthase (Prehm P, 1983), unlike other polysaccharides which are synthesized in the smooth endoplasmic reticulum of cells.

It is an important component of the early stages of wound healing. It has been demonstrated that wounded tissues express higher levels of HA than non-wounded tissues, but levels fall off after about day 5 (Bronson RE et al., 1987).HA levels are raised in foetal wounds and it has been suggested that these high levels may be one of the mechanisms for scar less wound healing observed in foetuses (Adzick NS et al., 1985b, Boyce DE et al., 1997, Krummel TM et al., 1987)

HA is a very hydrophilic molecule, therefore it creates a highly porous network into which cells such as fibroblasts migrate. The cells then degrade the matrix, the degradation products of HA have been observed to be potent stimulators of angiogenesis (Lees VC et al., 1995, Mast BA and Haynes JH et al., 1992b).

In the foetus, the less resistant HA-rich matrix could allow faster cell infiltration into the wound site, enhance cell proliferation, and influence the amount and type of collagen deposited, the nature of the fibrils formed, thus resulting in a more reticular pattern of collagen deposition. This may result in the scarless wound healing observed in the foetus.

In the adult, as the wound matures, levels of HA declines, and levels of other extracellular matrix proteoglycans such as chrondrotin-4-sulphate and dermatan sulphate increase to take its place (McCallion RL and Ferguson MWJ, 1996).

1.3.3 Collagen

This is a generic term for a family of closely related triple helical glycoproteins found in the extracellular matrix. About nineteen distinct collagen types have so far been categorised, and additional types are continuously been discovered (Gordon MK et al., 1992, Myers JC et al., 1994). They make up 25 – 30% of the body protein. Collagen type I is the commonest, constituting 90% of the total body collagen. Each collagen type is made up of three polypeptide α-chains, coiled into a left hand helix with the amino acid glycine at each turn, then twisted around each other into a left hand super helix (Stryer L, 1981).

There are nine collagen types, which are known to be important in the reparative process of wound healing: type I, III, IV, V, VI, VII, XII, XIV, XVII. The function of the more recently described collagen in cutaneous healing remains unclear. The more important ones, are types I, III, IV, VII..

Type I collagen is the major constituent of fibrous connective tissue, skin, tendon ligaments and bone. Early wounds however, as previously stated, contain relatively more type III collagen (Kurkinen M et al., 1980), which is replaced by type I collagen as the wound matures. Type IV collagen forms the scaffolding of the basement membrane, and Type VII collagen forms an essential component of the anchoring fibrils and hemidesmosomes. These link the epithelium to the underlying connective tissue, and defects in type VII collagen has been linked to the blistering skin disease – Epidermolysis bullosa (Burckner-Tuderman L, 1993).

Collagen is degraded in the wound environment by the by-products of bacteria and proteases acting as chemotactic agents for fibroblasts, neutrophils, and granulocytes.

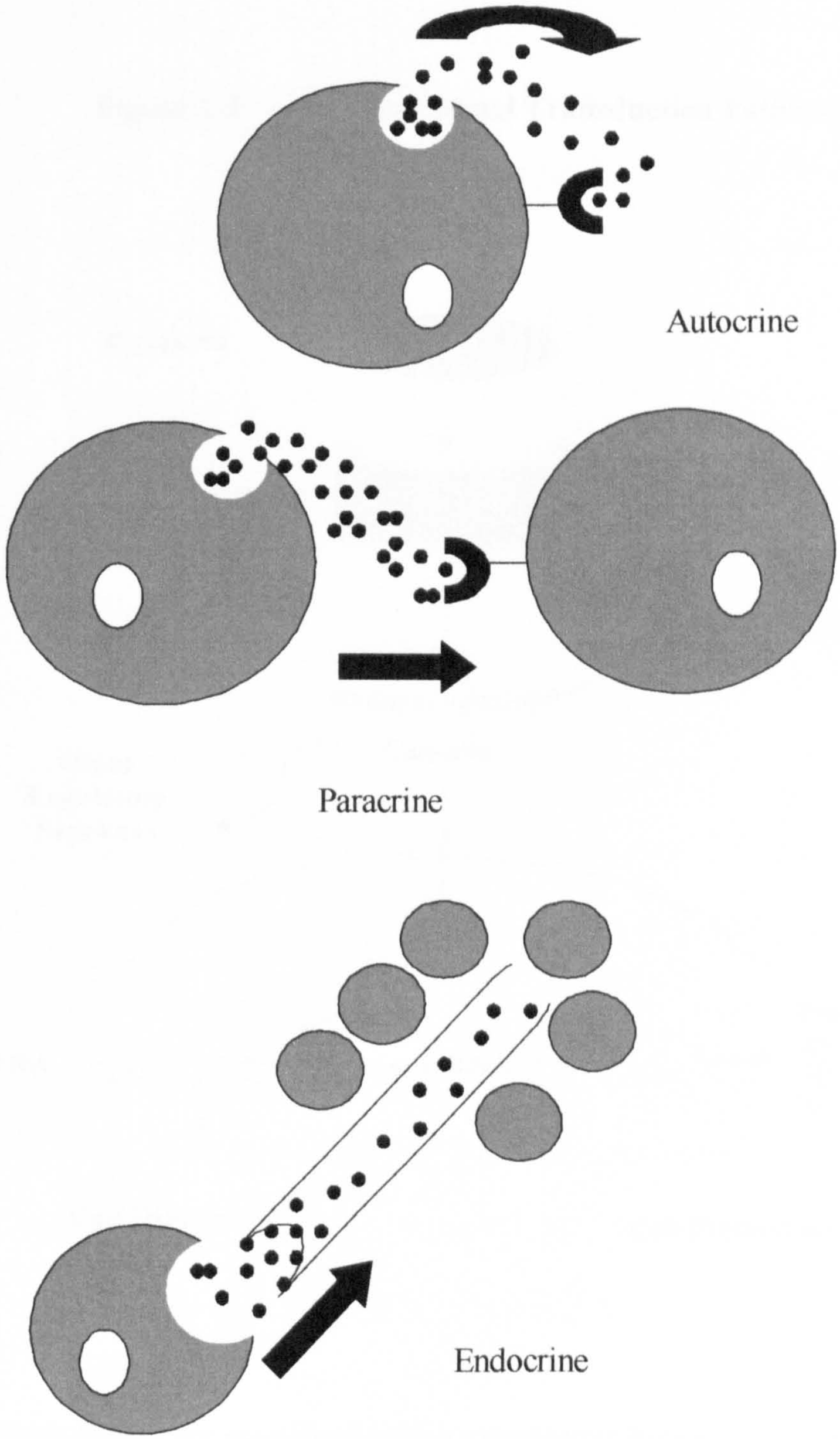
These cells in turn release proteases and cytokines regulating the various phases of wound healing (Postlethwaite AE et al., 1978, Werb Z and Gordon S, 1975).

Thus wound repair, in the extracellular matrix may induce changes in cell phenotype that in turn may induce matrix modification, creating a chain reaction until tissue stabilisation is achieved.

1.4 CYTOKINES

Cytokines are regulatory proteins and encompasses those families that are commonly referred to as growth factors, colony stimulating factors, interleukins, lymphokines, monokines, and interferons. The array of names for cytokines has risen because several different investigators discovered these regulatory proteins in different inflammatory and non-inflammatory cells, and the term cytokine is the most general definition. This definition is not restricted to the immunohaemopoietic system (Cohen S et al., 1974). Cytokines are secreted from various cells in the tissue and act locally in an autocrine (affecting the same cells that produce them), paracrine (acting on adjacent cells) or endocrine (acting on distant cells) way to control cell viability, proliferation, differentiation and death (Metcalf D, 1992) (Figure 1-2)

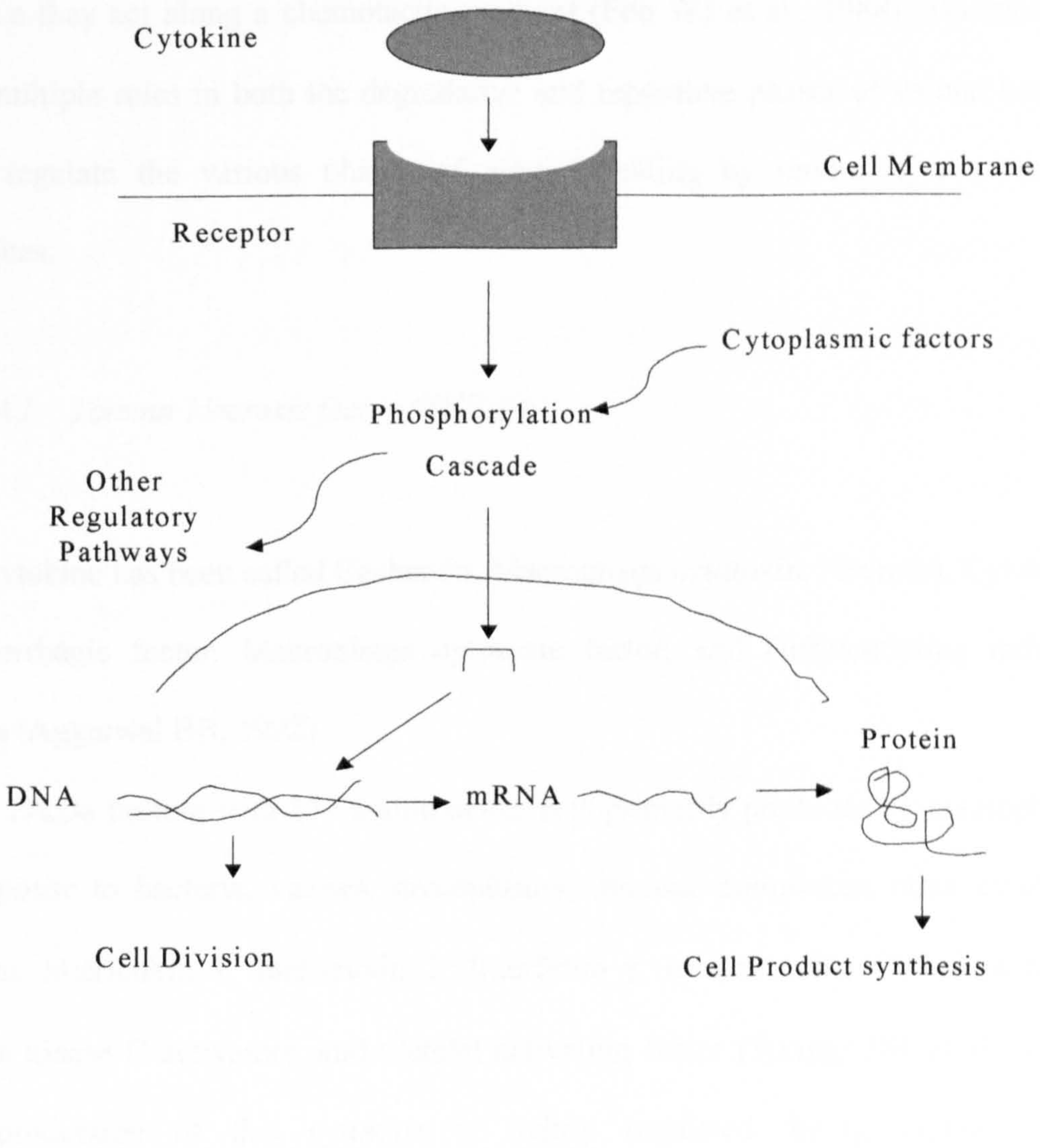
Figure 1-2 Mechanism of Cytokine Activity



Although the specific pathways, reactions, and functions vary for each cytokine, these processes are similar enough to be generalised in the following way. Activity is

initiated when a cytokine binds to specific, high affinity receptors on the cell membrane, followed by signal transduction (Trippel SB et al., 1996). (Figure1-3)

Figure 1-3 Cytokine Signal Transduction Pathway



Most growth factors are associated with a cytoplasmic kinase, which initiates a series of intracellular alterations, eventually leading to phosphorylation of proteins within the nucleus of the cell necessary for gene expression. The end result is increased

protein synthesis, changes in cellular activity, and/or proliferation of cells (Greenhalgh DG, 1996, Bennett NT and Schultz GS, 1993).

The cell surface receptors to which cytokines bind to are multimeric complexes. Binding activates various signal transduction pathways within the cell and result in an appropriate biological response in the target tissue (Dy M et al., 1999).

Cytokines are secreted in very small quantities and often directional to the responder cells i.e they act along a chemotactic gradient (Poo WJ et al., 1988). Macrophages play multiple roles in both the degradative and reparative phases of wound healing. They regulate the various phases of wound healing by producing an array of cytokines.

1.4.1 Tumour Necrosis factor (TNFα)

This cytokine has been called Cachectin, Macrophage cytotoxin, Necrosin, Cytotoxin, Haemorrhagic factor, Macrophage cytotoxic factor, and Differentiating inducing factors (Aggarwal BB, 1992).

It is a 17kDa protein with 157 amino acids. It is primarily produced by macrophages in response to bacteria, viruses, mycoplasma, immune complexes, other cytokines such as Interleukin 1, Interleukin 2, Interferon γ, tumour cells, Complement 5a, protein kinase C activators, and platelet activating factor (Spriggs DR et al., 1992).

The production of this cytokine is tightly regulated, being suppressed by Dexamethasone, Prostaglandin E_2, Transforming growth factor ß, Cyclosporin A, Interleukin 4, and Interleukin 6.

In vitro, TNFα inhibits the proliferation of certain tumour cells, stimulates the proliferation of fibroblasts, induces endothelial cell adhesion molecules, induces the

production of plasminogen activator plasminogen inhibitor, activates neutrophils, allows differentiation of myeloid cells, and induces the production of other cytokines.(Nacy CA et al., 1991)

In vivo, it is crucial in initiating the immune cascade during the host response to injury or bacteria. TNFα is involved in the recruitment and maturation of the cellular component of inflammation, which includes the up-regulation of cell-surface adhesion molecules that plays a vital role in the immune cell to endothelium interaction, thus facilitating neutrophil chemotaxis (Omann GM and Hinshaw DB, 1997, Moser R et al., 1989).

Its main effects include haemostasis, increased vascular permeability and proliferation. It also promotes many cellular metabolic events that increase the supply of nutrient substrates and acute-phase protein synthesis essential for wound healing (Fong Y and Lowry SF, 1996).

1.4.2 Interleukin-1 (IL-1)

This family of cytokines comprises of α, and ß, subgroups. They have been referred to as Catabolin, Endogenous pyrogen, Osteoclast activating factor, Lymphocyte activating factor, Leucocyte endogenous mediator, Fibroblast activating factor, and B cell activating factor.

The main source of IL-1 production are cells of the monocyte – macrophage lineage, namely activated macrophages, secreting IL-1 β, and human keratinocytes predominantly secretes IL-1α, although most cell types have the potential to produce this cytokine (Mizutani H et al., 1991). IL-1α is a 17kDa protein made up of 159 amino acids, and IL-1β is a 17kDa protein made up of 153 amino acids. IL-1

production is induced by a wide range of stimulants including bacterial products (eg.lipopolysaccarides from bacterial cell walls), complement components, other cytokines such as TNF, IFNγ, GM-CSF, and IL-1 itself. The synthesis of IL-1 is inhibited by Prostaglandins, and Glucocorticoids (Shaw RJ et al., 1990, Shaw RJ et al., 1991, Muller K et al., 1988).

IL-1 can affect a wide range of target cells and organs. It has been described as playing a central role in the effector phase of the immune and inflammatory responses (Dower SK et al., 1992a, Dinarello CA, 1991, Oppenheim JJ et al., 1986). IL-1 also exerts its effects by inducing the production of secondary cytokines such as IL-6, Colony stimulating factors and other chemokines (Raines EW et al., 1989, Qwarnstrom EE et al., 1993).

IL-1 activates endothelial cells in a pro-inflammatory, and pro-thrombotic way. This induces the production of tissue factor and platelet activating factor, down regulating the proteins C-dependent anti-coagulation pathway and the inhibition of thrombus dissolution.

In the acute inflammatory phase of wound healing, the main biological activity of IL-1 is to stimulate T-helper cells which are then induced to secrete IL-2 and to express IL-2 receptors on their surface. They also induce the production of acute phase proteins such as C-reactive protein, which has a role in the protection and antitoxic effects of the invading infection. IL-1 also acts directly on B-cells in promoting their proliferation and synthesis of immunogloblins (West MA, 1990). The production of collagenase and metalloproteinases is induced by IL-1, which also stimulates fibrobalsts to proliferate and synthesize collagen. Hence IL-1 may play a role in the remodeling phase of wounds.

1.4.3 Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen that has the ability to promote angiogenesis. It is a glycoprotein weighing 45000 kDA and exists as four isomers ranging from 121 to 206 amino acids (Leung DW et al., 1989, Houck KA et al., 1991).

The main function of VEGF is to promote the proliferation of vascular endothelial cells in both small and large vessels at dosages of ED_{50} 2 – 110pM (Ferrara N and Henzel WJ, 1989, Plouet J et al, 1994). In higher doses (ED_{50} 300 – 500pM), VEGF seems to induce monocyte chemotaxis by increasing the permeability of blood vessels. This was demonstrated by Connolly et al. in 1989, (Connolly DT et al., 1989,)

1.4.4 Transforming Growth Factor β (TGFß)

This is a general name for a family of naturally occurring polypeptides, which have multiple regulatory effects on cell proliferation and differentiation. (Cox D, 1995) Three isoforms exist in mammals – designated TGFβ1, TGFβ2, and TGFβ3.

TGFβ1 was originally purified from blood platelets, and TGFβ2 was purified from bone marrow. The biologically active TGFβ is a 25 kDa disulphide-linked homodimer polypeptide chain (Gitelman SE and Derynck R, 1994).

All TGFβs have receptors on virtually every cell in the body. TGFβ is chemotactic for macrophages, induces procollagen type I and fibronectin, and inhibits metalloproteinases (Overall CM et al., 1991). The presence of TGFβ results in an

increased deposition of collagen in cutaneous wounds (Pierce GF et al., 1992, Quaglino D et al., 1990) and has been shown to accelerate normal wound healing in a rat model (Martin P et al., 1992). Administration of TGFβ1 prior to wounding has also been demonstrated to accelerate healing. (Beck LS et al., 1993)

Although TGFβ3 has similar biological activities as its isoforms, it is probably more potent than TGFβ1 or TGFβ2 in stimulating neovascularisation, (Cox D.1995) but unlike the other forms, TGFβ3 has shown to reduce scar formation in rat incisional model and may have a role in the management of hypertrophic and keloid scars. (Shah M et al., 1992)

1.4.5 Basic Fibroblast Growth Factor (bFGF)

This family consists of at least nine homologous polypeptides. The most researched is the basic FGF (bFGF) which is a 18kDa heparin-binding, single chain polypeptide. However, 22 – 25kDa bFGF have also been isolated, but their functions are yet to be determined (Prats H, et al., 1989). It exhibits angiogenic effects by stimulating endothelial and smooth muscle cells. Inducing proliferation of epithelial cells and fibroblasts (Montansano R et al., 1994).

bFGF is a 146 amino acid polypeptide. An important characteristic of bFGF is its ability to bind to heparin and heparan sulphate which protects it from heat, and proteolytic degradation. This also increases the affinity of FGFs to bind to receptors on migrating and proliferating epidermal cells and hair follicle keratinocytes adjacent to the wound edge by 2-3 fold. (Rapraeger AC et al., 1991)

It has been demonstrated that bFGF increases granulation tissue in excisional wounds, but is sensitive to the partial pressure of O2 in the wound environment, as its effects are reduced or inhibited in ischaemic wounds. (Mustoe TA et al., 1991, Pierce GF et al., 1992, Mustoe TA et al., 1994)

1.5 MACROPHAGES IN WOUND HEALING

The term 'Macrophage' was first coined by Metchnikoff in 1884, which literally means big eater, when he demonstrated that rabbit and human leukocytes can engulf various bacteria, a process he called phagocytosis. In contrast, he called other phagocytic cells blood microcytes, later to be called polymorphonuclear leukocytes and granulocytes (Van Furth R et al., 1972). The role of humoral factors in the phagocytic process was not appreciated until 1903, when Almroth Wright first demonstrated that humoral factors called opsonins were essential for phagocytosis of bacteria (Van Furth R, 1992).

As previously described, the inflammatory phase of wound healing begins minutes after injury. The first type of cells to be attracted and mobilised at the site of injury are the neutrophils. These increase rapidly reaching a peak at 24 – 48 hours. It has been observed that depletion of circulating neutrophils in guinea pigs, has no significant effect on healing of experimentally induced wounds, and thus does not seem to play an essential role in the process of wound healing (Simpson DM and Ross R, 1971, 1972).

Monocytes emigrate into the injured tissue at about the same time as neutrophils, but as neutrophils are abundant in the circulation, they are the predominant cell type for the first few days after injury. As discussed previously in 1.2, monocyte influx into the wound environment is stimulated by selective chemoattractants, such as fragments of collagen, elastin, fibronectin, enzymatically active thrombin and TGFβ There they differentiate into macrophages. It used to be thought that the main functions of these macrophages are to remove and degrade injured tissue debris. However in 1975, Leibovitch and Ross, demonstrated that the combined depletion of circulating

monocytes and tissue macrophages in guinea pigs, resulted in severe retardation of tissue debridement and a marked delay in fibroblast proliferation and collagen deposition (Leibovitch SJ and Ross R, 1975). This indicated for the first time that macrophages play a vital role in the orchestration and execution of both the degradative and the reparative phase of wound healing. Hunt et al, in 1984 demonstrated that wound inflammatory cells harvested from subcutaneous wounds stimulated angiogenesis, fibroplasia, and collagen synthesis in the cornea of rabbits compared to controls and concluded that wound macrophages released substances that caused these responses (Hunt TK et al., 1984). Reduced oxygen tension (hypoxia of 5 – 50mmHg) and high lactate concentration (10 – 15mM) have been shown to stimulate macrophages to produce growth factors that result in angiogenesis, thereby initiating neovascularisation in the wound space (Hunt TK et al., 2000).

Macrophages therefore seem to regulate the inflammatory and the reparative phases of wound healing in animal models. The understanding of the role of macrophages in human wound healing will be discussed in this thesis.

1.5.1 The monocyte – Genesis and Differentiation

Monocytes are derived from a pleuripotential stem cell, which are thought to reside in the bone marrow and differentiate into lymphoid stem cells, myeloid stem cells or replicate into pleuripotential stem cells. The myeloid cell line gives rise to progenitor cells of the of the megakaryocytic, erythroid and the granulo – monocytic cell lines in response to Interleukin-1 (IL-1), Interleukin-3 (IL-3) and Interleukin-6 (IL-6) (Ogawa M, 1993). In response to specific growth factors such as the granulocyte-macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor(M-

CSF) the granulo – monocytic cells give rise to neutrophils and monoblasts (monocyte precursor cells). The first cells of the monocyte –macrophage system that can be detected in the blood are the promonocytes. The maturation of these cells to monocytes require the presence of M-CSF. Monocytes are different from their precursors due to their inability to replicate, and develop most of the lysosomal system which enhances phagocytic activity (Hamilton JA, 1993).

The monocytes then leave the bone marrow and travel to the injured tissue via the blood stream. They move into the injured tissue via a chemotactic response to tissue hypoxia, degradation products resulting from the injured tissue and neutrophils. (Postlethwaite AE and Kang AH, 1976. Clark RAF et al., 1988, Wolpe SD and Cerami A, 1989) Entering the tissue, they differentiate to macrophages. They undergo further development by growing, increasing their lysosomal contents, increasing the number and size of their mitochondria. This is in preparation for increased energy expenditure, production of various cytokines and growth factors. However, the mechanisms responsible for driving the differentiation remain elusive. Several serum constituents have been implicated, including 1α,25-dihydroxyvitamin D_3 (Proveddini DM et al, 1986) and fibronectin (Wright SD et al, 1983). The fibronectin theory stems from the observation that circulating blood monocytes have a fibronectin receptor, the β1 integrin. Binding of fibronectin stimulates a host of changes within the monocyte including, the phagocytosis, and secretion of plasminogen activator and elastase, functions associaied with inflammatory macrophages (Hosein B et al., 1985). The story however is not fully understood, as it is possible that other cytokines in the wound environment may influence monocyte differentiation in an autocrine or paracrine fashion. It may therefore be the result of a combined stimulation.

1.5.2 Monocyte Influx vs. Local Production

Several investigations have shown that resident tissue macrophages have the ability to replicate *in situ*, and there is an argument that circulating blood monocytes may not be required to replenish the tissue macrophages. This however depends on the type of tissue. Pulmonary macrophages for example have been shown to proliferate locally. Systemic hydrocortisone administration or bone marrow irradiation seems to have little effect on the concentration of the alveolar macrophage population and local irradiation of the thorax seems to deplete the alveolar macrophage population (Lin H et al., 1982, Sawyer RT et al., 1982, Tarling JD and Coggle JE, 1982).

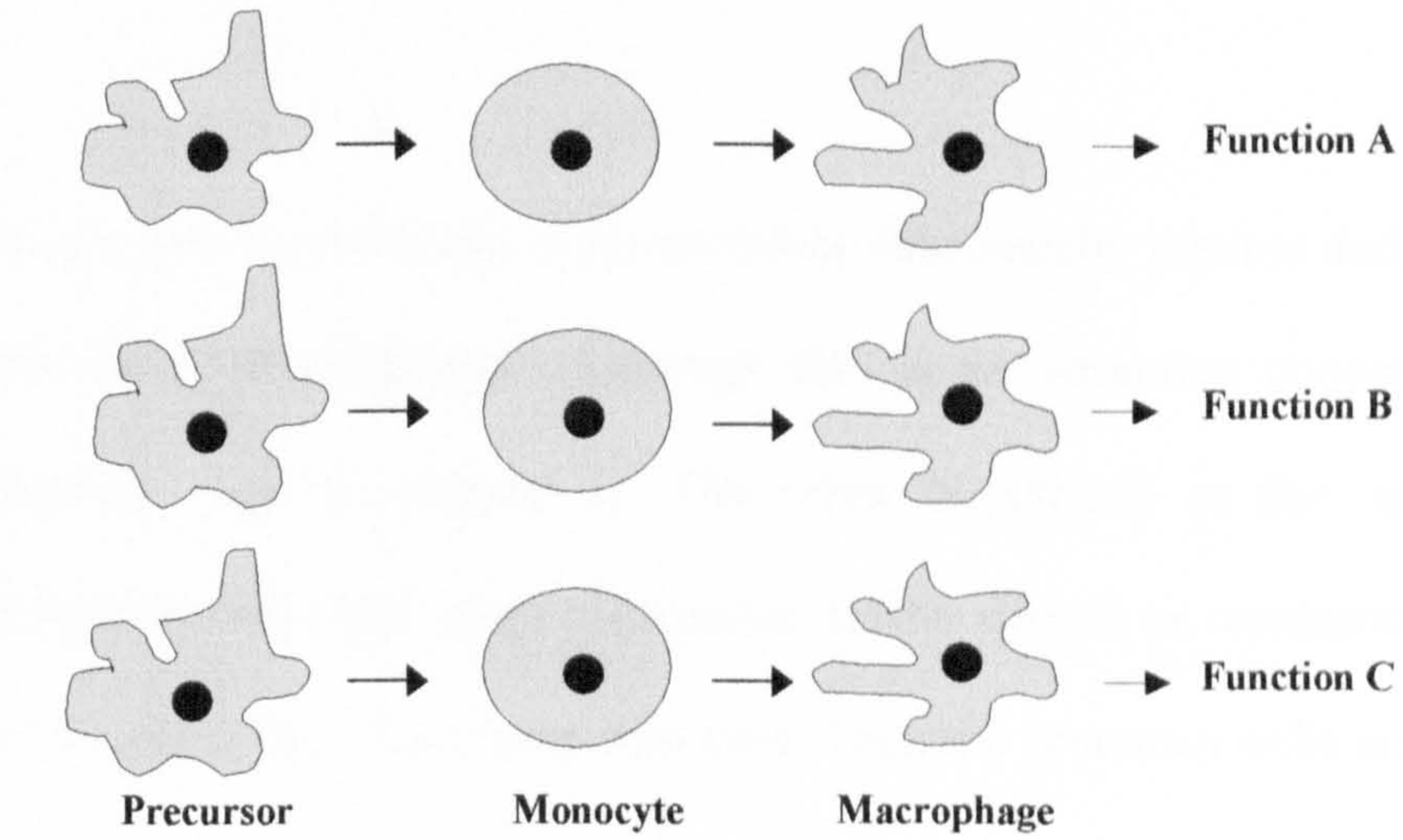
Skin contains very few macrophages, but during inflammmation, the numbers increase dramatically. Experiments by Leibovitch and Ross in 1975, seems to suggest that macrophages found in the tissue are derived from blood monocytes. This suggestion is also backed up observations made by Stewart et al., in 1981. By observing the sex chromosome markers of macrophages emigrating from explants of a 5-day skin wound in a girl who recently received a bone graft transplant from her brother, it was found that the macrophages bore the karyotype of the donor ie. The macrophages had the male XY chromosomes (Leibovitch SJ and Ross R, 1975, Stewart RJ et al., 1981).

1.5.3 Phenotypic Differentiation

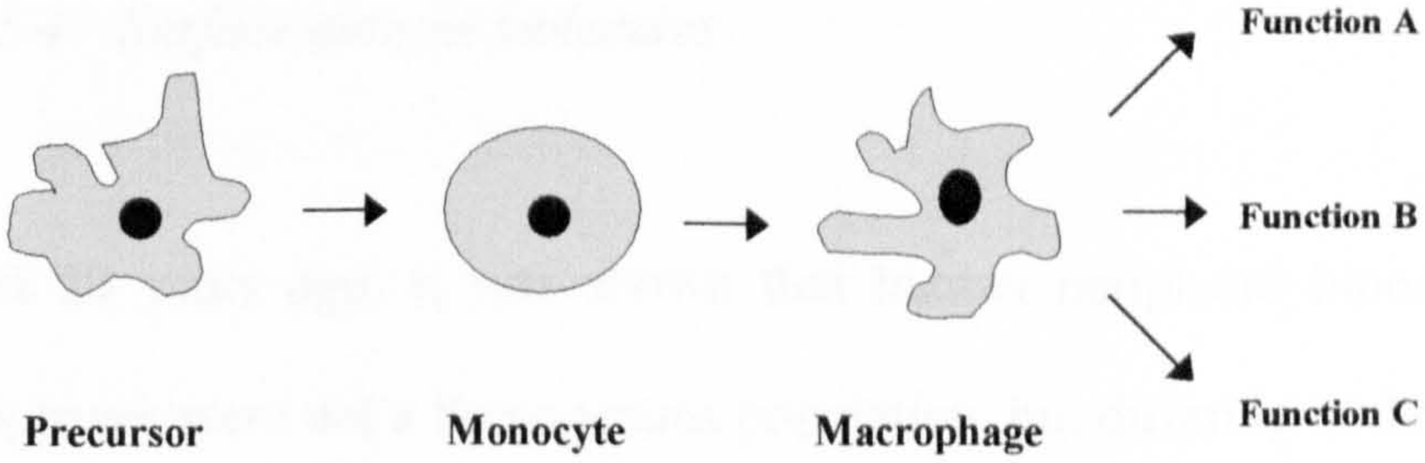
Macrophages contribute to a variety of functions in the degradative and reparative phases of wound healing and other aspects of host defences. These include the recognition, elimination of cells infected by viruses, intracellular parasites, and

presentation of antigens to the immune system. Two general hypotheses have been generated to explain the heterogenic roles exhibited by the macrophages. This is depicted in figure 1-4.

Figure 1-4 Two Hypothesis of Macrophage Phenotype Differentiation



Hypothesis 2 - Adaptive function of macrophage diversity



The first is that the diversity is generated by functionally distinct and committed sub-populations of macrophages. Although this is an attractive concept there is little experimental data to support it. The other hypothesis is that macrophages are pleuripotential cells that adapt themselves to the stimuli or conditions that prevail at the site to which they have been attracted. Thus the precursor cells are a homogenous group of cells (Gordon S et al., 1995).

The adaptation theory was first described by Russell et al in 1977 and Ruco and Meltzer in 1978. They demonstrated that peritoneal macrophages could differentiate to express new functional activities and cytocidal activities in response to various immune and bacterial stimuli *in vitro*. The induced macrophages express a number of gene products that collectively contribute to the altered functional activity (Russell SW et al., 1977, Ruco LP and Meltzer MS, 1978).

Some of these macrophage products are inducible nitric acid synthase (Ding AH et al., 1988, Lowenstein CJ et al., 1992), complement components (Riches DWH and Stanworth DR, 1982a) and interferon-β (Riches DWH and Underwood GA, 1991).

1.5.4 Surface antigen molecules

Almost 20 years ago, it was shown that human peripheral blood monocytes and macrophages were not a homogenous population, but differing in their phenotype and function.

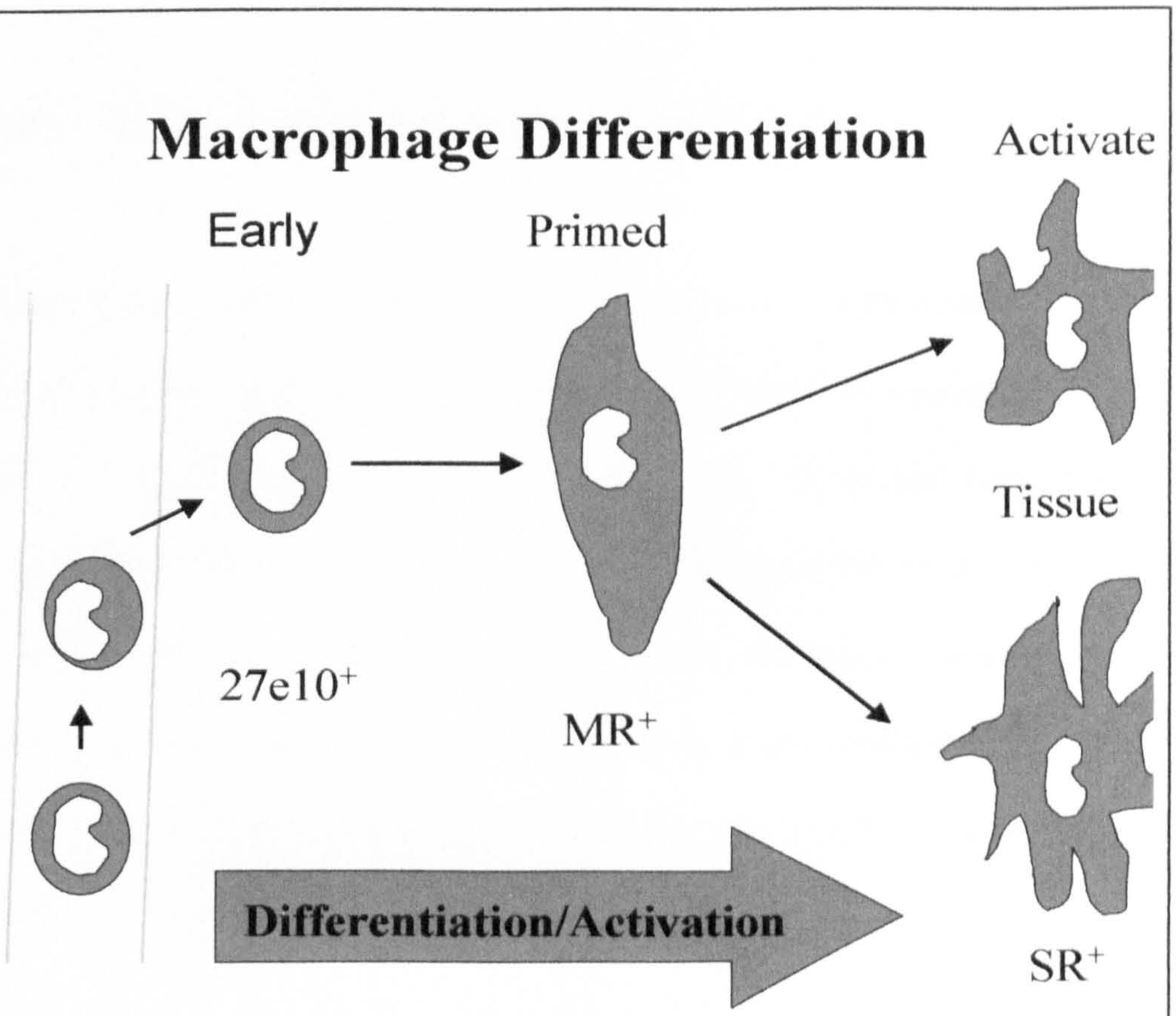
The earliest isolation procedures utilised gradient centrifugation and counterflow centifugation techniques (Figdor CG et al., 1982, Weiner RS and Mason RR, 1984). These separated the macrophage subsets by size and density. Akiyama et al. defined these as a major population of regular macrophages and a minor subset of intermediate macrophages (Akiyama Y et al., 1983). Regular macrophages were characterised by larger size, higher expression of peroxidase activity, and higher antibody-dependent cell-mediated cytotoxity. The intermediate macrophages on the other hand, produced less peroxidase activity, but could be more easily mobilized from extracellular reservoirs.

More recently, macrophages were characterised on their differences in the surface marker expression. Monocytes and macrophages express several well known surface molecules. It was also found some of these molecules were also found on other haemopoietic cells, fibroblasts, endothelial and epithelial cells, but there are still few examples of macrophage-specific surface markers (Zhang DE et al., 1994, Xie Q-W et al., 1993, Feinman R et al., 1994).

There is little evidence to suggest the existence of specific surface markers present on monocytes alone. However, there are certain markers of high restricted specificity found on macrophages at different differentiation stages in the local environment of acute and chronic wounds. With the use of specific monoclonal antibodies, these membrane molecules function as excellent markers to study the differentiation and distribution of sub-population of macrophages. Experiments on animals, to date, have demonstrated the existence of an array of macrophage sub-population in murine tissue models (Gordon S et al., 1992).

There is also some evidence to suggest that human macrophages also exhibit an array of surface markers at different differentiation stages. (Grage-Griebenow E et al., 2001) (Figure 1-5)

Figure 1-5 Proposed Pathway of Macrophage Differentiation



Schreiber et al in 1991, has shown that as monocytes differentiate into macrophages, they progressively develop phenotypic and functional characteristics pivotal to host defence. At the same time they also express specific surface markers. This could be a convenient way to monitor maturation of macrophages in wounded tissue (Schreiber S et al., 1991).

1.5.5 Macrophage Antigens

1.5.5.1 The pan-macrophage marker - CD68

This is a 110 kDa glycoprotein that stains macrophages in a wide variety of human tissues, including peripheral blood monocytes, Kupffers cells and macrophages in the red pulp of the spleen, lamina propria of the gut, lung alveoli and bone marrow (Pulford KAF et al., 1990, Naksted B et al., 1989, Athanasou NA et al., 1986). It is therefore considered as a pan-macrophage-marker detecting monocytes and macrophages at all stages of differentiation. Therefore it can function as a valuable marker for monocytes and macrophages in wounded tissues which are composed of a variety of inflammatory cells.

1.5.5.2 The early macrophage marker - 27e10

The 27e10 antigen is formed from two non-covalently linked complexes of the migration inhibition factor related proteins (MRP) 8 and 14. It has a molecular weight of 22kDa (MRP 8 has a molecular weight of 8kDA and MRP 14 has a molecular weight of 14kDa). This antigen has also been referred to as the Cystic Fibrosis antigen, CalgranulinA/B, the L1 antigen and calprotectin to name but a few (Wilkinson MM et al., 1988, Clark BR et al., 1990, Dale I, Fagerhol MK and Naesgaard I, 1983, Steinbakk M et al., 1990). The 27e10 antigen belongs to a group of proteins called the S100 family and is calcium dependent for most of its activities. Calcium is important in the release of enzymes, cytoskeleton membrane interactions, regulation of cell differentiation and cell cycle progress (Odink K et al., 1987,

Kligman D and Hilt DC, 1988, Heizmann CW and Hunziker W. 1991). Calcium ions seem to play a regulatory role in the transcriptional stage of the 27e10 antigen synthesis. Excess Ca^{2+} switches off the production of the antigen by *de-novo* synthesis of suppressor proteins. Antigen production is upregulated by vitamin D_3. After synthesis, the 27e10 antigen is extruded to the cell surface via a novel pathway requiring an intact micro-tubule network following the activation of the protein kinase C system (Roth J et al., 1994, Roth J et al., 1993, Warner-Bartnicki AL et al., 1993. Rammes A et al., 1997).

In vitro, IL-4 and IL-10 has been shown to inhibit the expression of 27e10 antigen on the surface of early macrophages (Lügering N et al., 1997).

This antigen has been shown to be present in pro-inflammatory, early macrophages which are found in acutely inflamed tissue but absent in mature tissue macrophages (Zwadlo G et al., 1988).

$27e10^{+ve}$cells produce large amounts of inflammatory mediators such as prostaglandin E_2, Tumour necrosis factor (TNF), and interleukin-1 (IL-1) in the wounded tissue, thus perpetuating the stimulus for influx of other inflammatory cells into the wound. (Zwadlo G et al., 1986).

Hence 27e10 monoclonal antibody can be used as a convenient marker to identify early inflammatory macrophage in healing and non healing wounds.

1.5.5.3 The primed macrophage marker - Mannose Receptor (MR)

The macrophage mannose receptor (also known as the mannose – fucose receptor) is a 175 kDa membrane glycoprotein whose presence on the macrophage membranes was first revealed as a result of *in vivo* clearance experiments with lysosomal hydrolases (Stahl PD, 1990). This receptor recognises glycosylated molecules with terminal mannose, fucose, or N-acetylglucosamine moieties and efficiently internalises soluble and particulate ligands through endocytosis and phagocytosis, respectively (Pontow SE et al., 1992). The expression of the receptor is highly regulated and correlates with the functional state of the monocyte/macrophage. Monocytes do not express mannose receptors and it has been shown that primed macrophages express higher levels than activated macrophages. Increased receptor expression is mediated by 1,25 dihydroxyvitamin D_3 (Clohisy DR et al., 1987), Prostaglandin E (Schreiber S et al., 1990), dexamethasone (Shepherd VL et al., 1985), IL-4 (Stein ML et al., 1992), LI-13 and IgG2a (Doyle AG et al., 1994, Schreiber S et al., 1991). In contrast, MR expression is down-regulated by interferon-γ, immune complexes and bacterial lipopolysaccharides (Noorman F et al., 1995, Imber MJ et al., 1982, Mokoena T and Gordon S, 1985, Shepherd VL et al., 1990.).

It has been suggested that the mannose receptor plays a role in the uptake of antigen followed by antigen presentation by dendritic cells, Langerhan cells, and macrophages *in vitro* (Sousa R et al., 1993, Muchmore AV et al., 1990). However, Noorman et al. in 1997 showed that there are no MR^{+ve} dendritic cell families. He suggested that macrophages exist in two stages of differentiation. Primed macrophages exhibiting the mannose receptor with its ability to capture and process the antigens, once

stimulated become activated during antigen processing lose the receptor and produce cytokines to stimulate the T cells. (Noorman F et al., 1997)

In summary, MR^{+ve} cells identify primed macrophages.

1.5.5.4 Tissue macrophage marker - Sialoadhesin Receptor (SR)

Tissue macrophages are distributed throughout the organs and tissues of the body, where they are thought to play a central role in both innate and specific immune responses and in the maintenance of normal homeostatic mechanisms. The precise function of macrophages within specific microenvironments such as the bone marrow is poorly understood. It appears that these cells maintain a discrete population of surface receptors, which are specific for their particular anatomical/functional location and which appear to be controlled in a spatially and temporally precise manner. The way in which tissue macrophages interact with other cells and with components of the extracellular matrix is believed to be regulated via lectin-like domains, indicating an important role for carbohydrate structures in cell recognition (Speert DP et al., 1988, Bevilacqua MP et al., 1989, Johnston GI et al., 1989).

Sialoadhesin is a macrophage restricted plasma membrane receptor of 185kDa, and is a member of the newly recognised family of sialic acid binding lectins called siglecs. It is the largest member of the sigelic family. Sialoadhesin specifically recognises the oligosaccharide sequences NeuAcα2,3Galβ1,3 GalNAc and NeuAcα2,3Galβ1,3(4)GlcNAc in either glycoproteins or glycolipids. This may function as important recognition molecules in cellular interactions between stromal tissue, macrophages, haematopoietic and lymphoid tissues, thus playing a role in wound healing (Crocker PR et al., 1991, Kelm S et al., 1994).

In bone marrow, SR^{+ve} cells are localised to areas of contact between the resident stromal macrophages and the developing granulocytes. In the spleen these cells are found in the marginal zones, in the subcapsular sinus and medulla of lymph nodes. During T cell-mediated autoimmune diseases and chronic inflammation, sialoadhesin is also expressed by the infiltrated macrophages in the affected tissues (Dijkstra CD et al., 1985, Dijkstra CD et al., 1987, Noble B et al., 1990).

On rat macrophages, sialoadhesin expression is directly induced by glucocorticoids and the glucocorticoid-dependent sialoadhesin expression is enhanced by interferon-β, interferon -γ, IL-4and LPS (Van den Berg TK et al., 1996), but in the mouse, IL-4 has been described to selectively inhibit or down regulate the expression of SR. This inhibitory effect can be modified by the presence of other cytokines in the tissue microenvironment (McWilliam AS et al., 1992). This is opposite to the expression of the MR in the presence of IL-4, where it has a stimulatory effect and an observable increase in MR^{+ve} cells within the wounded tissue. The effect of IL-4 in human macrophages is not yet elucidated, but the authors believe that it may play a vital role in the regulation of SR^{+ve} tissue macrophages within the wounded tissue.

The earliest identified function of SR^{+ve} cells was the ability to agglutinate unopsonized sheep and human erythrocytes resulting in rosettes (Crocker PR and Gordon S, 1986). More recently Crocker et al have demonstrated a cell-cell binding hierarchy with a strong preference for cells of the granulocytic lineage. In rank order, he showed that of SR^{+ve} cells bound to neutrophils with the greatest avidity, followed by bone marrow cells, then blood leukocytes, then lymphocytes and weakly with thymocytes (Crocker PR et al., 1995). *In vivo,* the adhesion of granulocytes to SR^{+ve} cells may influence the function of the granulocytes and their turnover. For example, it has been demonstrated that the respiratory burst activity of granulocytes can be

suppressed by the binding of SR^{+ve} cells to granulocytes, thus preventing the undesired tissue damage caused by activated granulocytes (Cassidy LF et al., 1989). It has also been observed that senescent neutrophils are cleared from the circulation by SR^{+ve} cells in the reticuloendothelial system and *in situ* at sites of inflammation (Perry VH et al., 1992).

Thus SR^{+ve} macrophages, through their direct receptor-cell interaction with other cells of the inflammation such as the granulocytes, lymphocytes and thymocytes, may play a vital role in influencing the behaviour of these cells in chronic inflammation. This is yet another distinct function of a specific subset of macrophages rather than secreting cytokines when the macrophages become activated.

In this study SR identifies tissue macrophages in wounds.

1.6 HUMAN WOUND HEALING MODELS

Most of today's understanding of the mechanisms of wound healing, is based on animal models. It is known that human skin is unique and highly variable according to age, sex, race, and region of the body. There is no single model that can be utilised, to represent all the wound types seen in humans (Montagna W. 1971, Butterworth RJ, 1992).

Another problem is that there are currently no good animal models to represent chronic wounds such as venous leg ulcers, diabetic ulcers or pressure sores. Manna et al in 1982, attempted to evaluate guinea pig skin as a chronic wound model, eg. a venous leg ulcer, by inducing a chemical injury. They injected sodium tetradecyl sulphate into the flank skin of the guinea pig to mimic a sloughy ulcer, but found that these wounds healed in two weeks. This was quicker than venous leg ulcers, as they could not sustain venous hypertension or other microvascular abnormalities that are seen in venous leg ulcers and hence severely limited its value as a chronic wound model.(Manna V et al., 1982)

Another problem in creating chronic animal wound models is the definition of a 'chronic' wound. There is no definite definition of a chronic wound, but in humans this has been defined by the Wound Healing Society as a wound that has shown no signs towards healing in 8 weeks (Franz MG et al. 2000). Despite this, animal models do allow large numbers of similar wound environments to be studied. It is possible, on occasion to fill gaps in our knowledge of the pathogenesis of human disease by a close study of morphologically similar ones in animals (Scott GBD, 1976). An ideal animal species suitable for predicting response in humans should resemble skin structure and react similarly to a wide range of stimuli. In this respect, many animal

models were composed of 'hairless' strains of mammals, such as bald strains of rats, mice, rabbits, and dogs.

The subject of using animal models is controversial and ethical debates continue in every continent. Designing experiments requires careful forethought, animal models are time consuming, expensive, and the results can rarely be fully extrapolated to humans. However, animal models still provide invaluable information for the human wound healing response, but one must interpret the findings with caution (Chulakadabba A and Sheraman CP, 1999).

Butterworth studied granulating wound tissue in 26 patients using a 4mm punch biopsy, taken under local anaesthetic, and concluded that human tissue provided a more accurate and clinically relevant model. This raised fewer ethical objections than work on animal models. Informed consent for participation into study is also easier to obtain from individuals (Butterworth RJ, 1992).

For the reasons stated above, human models may be preferable in wound healing research, especially if it is directed to address problems in clinical practice.

1.6.1 The acute wound model: The pilonidal sinus (PNS) excision site

The term pilonidal sinus originates from the Latin words *nidus* for nest, *pilus* for hair and *sinus* for connections to the skin (Edwards MH, 1977). There is a male preponderance of 1.5:1 especially in hirsute men. The evidence for the condition as acquired is supported by its rarity in children and its similarity to the condition seen between the fingers of barbers, due to repeated contact with hair. In the natal cleft, it is postulated that the skin follicles are enlarged due to the shearing action of the

buttocks and it is the implantation of foreign hair into these follicles which subsequently becomes infected (Brearly R, 1955, Bascom J, 1980).

The infected area swells and occludes the follicular opening, rupturing into the subcutaneous fat, and eventually develops a sinus track to the skin (Allen-Mersh TC, 1990)

Numerous treatments exist, but none are perfect, judged by the outcomes of primary healing and recurrence of disease. Methods such as brushing of the track, laying open of track, primary closure, and asymmetrical skin incisions in an attempt to flatten the natal cleft to reduce buttock friction have all met with degrees of success (Lord PH, 1965, Clothier PR and Haywood IR, 1984, Karydakis GE, 1973)

The treatment of pilonidal sinus disease at the University Hospital of Wales, Department of Surgery, has been to widely excise the affected area, allowing the wound to heal by secondary intention, in the belief that this will lead to a lower incidence of recurrence of the disease (Marks J et al., 1985).

Thus the pilonidal sinus excision site forms an ideal acute wound model for study.

1.6.2 Chronic wound model: The chronic venous leg ulcer (VLU)

Chronic wounds as is their nature are more difficult to heal and sometimes harder to maintain complete closure. Conditions such as impaired venous drainage, impaired arterial supply, metabolic abnormalities, persistent mechanical forces, neurogenic defects and genetic disorders impairs the body's ability to repair tissue damage (Margery HD et al., 1992).

Venous leg ulcers affect about 1% of the population, increasing to around 3% in those 65 years or older (Callam MJ, 1992).

The most common leg ulcers seen in UK clinics are venous (70-80%) (Callam MJ et al., 1987) or ischaemic in origin (20-30%) (Callam MJ et al., 1987)

The financial cost of venous leg ulcer in Britain has been estimated to be between £294 – 650 million per year, depending on the variation in practice, and the location of provision of care (eg. Community or out-patient clinic) (Price P et al., 2000).

1.6.2.1 Aetiology of Venous Leg Ulcers

Venous reflux due to valvular incompetence is the main trigger for local changes in the microcirculation. This results in an increase in the ambulatory venous pressure in the subcutaneous veins of the lower legs and ankles, venous hypertension (Partsch H, 1995). Incompetent valves result in the failure or partial failure of the calf muscle pump, and possibly the foot pump plexus affecting both the macro- and micro-circulation, resulting in venous hypertension (Stemmer R et al., 1980, Creutzig A et al., 1994). There are three major theories of how this incompetence results in venous ulceration.

1.6.2.1.1 *The Fibrin Cuff/ Growth Factor Entrapment Theory*

When the sub-epidermal capillary bed is examined histologically in patients with venous ulcers, there is dilatation of the horizontal sub-capillary venous plexus with grossly coiled, elongated capillary loops and a reduction in the number of capillaries supplying the epidermis (Fagrell B, 1973). Fibrinogen, α_2 macroglobulins and other macromolecules leaks out from these dilated capillaries into the dermis of the wound, forming a peri-capillary fibrin cuff and this is thought to be responsible for the

reduced diffusion of oxygenated blood to the tissues (Burnand KG et al., 1982, Burnand KG and Browse NL, 1982, Stacy MC et al., 1988). Falanga and Eaglestein hypothesised that these macromolecules 'trap' growth factors or other homeostatic substances, thus rendering them unavailable for wound healing which occurs at the wound site (Falanga V and Eaglestein WH, 1993).

Their hypothesis stems from experiments performed by Higley et al. who characterised fibrinogen and α_2 macroglobulins within venous leg ulcers. α_2 macroglobulins are also known to be a binding protein for growth factors (Higley H et al., 1992).

However, the trap may not bind all the growth factors, but might alter the concentration or orderly sequence of the action of growth factors. The excess of macromolecular burden within the dermis may also bind to normal matrix components and interfere with their function in healing.

1.6.2.1.2 *The Leucocyte Entrapment Theory*

Venous hypertension due the failure of the valves, results in a reduction in the pressure gradient between the arteriolar and venular end of the capillaries in the leg. This causes a reduction in the velocity of the blood within these capillaries. This may lead to an increase in the adherence of blood cells to the endothelium of the capillaries releasing inflammatory mediators such as ICAM-1 and VCAM-1(Coleridge Smith PD et al., 1988). This may result in the obliteration of functioning capillary loops in the skin aggravating ischaemia leading to ulceration (Thomas PRS et al., 1998).

1.6.2.1.3 *The Microangiopathy Theory*

Using intravital capillaroscopy, it has been demonstrated that some of the capillaries are occluded by microthrombi or exhibit long intra-capillary stasis. This can in turn reduces nutrition and oxygenation of the skin, predisposing to ulceration. Therefore the functional capillary density is much less than the anatomical density (Steins A et al., 1999)

In consideration, probably all these factors play a role in the pathogenesis of venous leg ulcers.

The venous leg ulcer is therefore, a prime example of a chronic wound and can be used as a convenient chronic wound model.

Clinically these patients usually exhibit as varicosities in the lower limb, a brownish flaky appearance to the skin around the malleoli, termed lipodermatosclerosis, and minor trauma to this area results in a superficial ulcer which can pose a challenge to heal.

1.7 TISSUE ENGINEERED DERMAL REPLACEMENT - DERMAGRAFT®

Chronic wounds like venous leg ulcers can be difficult to heal within an acceptably short time, and in some patients are harder to maintain healed. Many of these patients are elderly, have multiple medical problems, poor diet, and poor social circumstances. Therefore effective treatment should encompass all these aspects. When possible, medical problems should be corrected, nutrition improved, and local wound care should be considered.

Traditionally the treatment of venous ulceration has been conservative: wound care, surgical correction, elevation and compression (Falanga V, 1993). Wound care in the form of sharp debridement to remove any dead tissue within the wound, topical dressings to maintain a moist, but not a saturated wound environment, and a balanced frequency of wound dressing changes, which will prevent saturation, but not disturb the healing wound un-necessarily (Brem H et al., 2004). Systemic antibiotics are also sometimes used to treat significant wound infection. Surgical correction of the underlying venous insufficiency in a small number of patients has also been of some benefit. In a recent study, evaluating surgical correction of isolated superficial venous reflux in ulcerated legs reported 12 and 24 week healing rates of 50% and 72% for operated legs and 62% and 74% for non-operated legs. However, these authors noted a high level of recurrence of 28%, 30%, and 44% for non-operated legs when compared to 14%, 20% and 26% for operated legs at 1,2,and 3 years respectively (Barwell JR et al., 2000).

The most effective treatment intervention for venous leg ulcers caused by venous hypertension is the application of compression bandages. However the mode of action

of compression therapy is still unclear. There is a large body of evidence that associates venous ulceration with high ambulatory venous pressures in the subcutaneous veins of the lower legs arising from valvular incompetence. This disrupts the local blood circulation, both the macro and micro-circulation, with an increase in the micro-circulation (Anning ST. 1952, Creutzig A et al., 1994). The application of compression therapy is thought to correct or improve this incompetence (Christopoulos DC et al., 1995, Krishnamoorthy L and Melhuish JM. 2000). Even with optimal care and follow-up, a recurrence of venous ulcers occurs in 29 – 59% of patients (Kowallek DL and De Palma RG, 1997).

Researchers over the years have tried numerous methods to promote healing. These have included skin grafting (skin transplant from one region to another), but this carries with a degree of morbidity, especially if the initial wounds are large (Eaglstein WH and Falanga V, 1998). Allografts, (skin transplant between two unrelated individuals of the same species) on the other hand, pose ethical consideration and entails problems with graft rejection (Navasaria HA et al., 1995). The problems associated with autogenic, allogenic and xenogenic (skin transplant between two species) skin substitutes have prompted the search for alternatives that would be widely available, and have properties closely resembling that of normal skin.

Modern attempts to develop human skin replacements began in the early 1960s with the advancement of tissue engineering (Rooman RP and Rosseuw D, 1992). Rheinwald and Green are often referred to as the pioneers of tissue engineering. They overcame the limitations of propagating mammalian cells by cultivating human epidermal cells enabling mass culturing of these cells (Rheinwald JG and Green H, 1975).

Major technical advances in tissue engineering over recent years have made it possible to culture human keratinocytes in laboratory conditions. One of the major problems with the use of cultured keratinocytes is the increase in wound contracture. Their have however been more promising results with the incorporation of dermal components to the keratinocyte cultures. This seems to improve the take and reduce contraction of the grafts (Couno CB et al., 1987, Odessey R, 1992). Several commercially available dermal substitutes have been shown to increase adherence of cultured keratinocytes to the dermis. Thes include Apligraf™, Alloderm™, and Integra™, which provide the dermal component to increase adherence of CEA (Falanga V et al., 1998, Wainwright D et al., 1996, Heimbach D et al., 1988, Freedlander E, 1998, Pomahac B et al., 1998, Hansborough JF and Franco ES, 1998). More recently, a living human dermal replacement, Dermagraft® [DG] has been developed (Advanced Tissue Sciences, Inc. USA, La Jolla CA) that can be permanently implanted to mimic the patient's damaged dermis thus facilitating healing.

DG is a bioengineered human dermis, consisting of neonatal dermal fibroblasts. Human fibroblast cells are harvested from neonatal foreskins, which are discarded after circumcisiom. The fibroblast cells are then seeded onto a three dimensional bioabsorbable scaffold made of polyglactin. As the fibroblasts proliferate within the scaffold, they secrete human dermal collagen, fibronectin, glycosaminoglycan, growth factors and other proteins, producing a dermal matrix. This results in a metabolically active dermal tissue with the structure seen in the papillary dermis of newborn skin. It has been shown that a single donor foreskin can provide enough cells to produce 250,000 square feet of DG (Naughton GK et al., 1997).

To ensure safety, maternal blood and cultured cells are extensively tested throughout the manufacturing process for known pathogenic agents such as Human Immunodeficiency Virus (HIV), Human T-cell Lymphotrophic Virus (HTLV), Cytomegalovirus (CMV), Herpes Simplex Viruses (HSV), and Hepatitis viruses (Naughton GK et al., 1997).

After manufacture, DG is stored at -70°C until used. The metabolic activity is assessed before and after cryopreservation as DG is designed as a living bioactive product (Mansbridge J et al., 1988).

DG is shipped in dry ice to the clinical site. Prior to implantation, the product is thawed, rinsed in sterile saline three times, cut to the appropriate wound size and placed onto the wound bed. The metabolically active fibroblasts deliver an array of growth factors, which are key to neovascularisation, epithelial migration, and differentiation and facilitates the implantation of DG into the patients' wound bed.

It has been shown that no immunological response, in the form of tissue rejection, was demonstrated when evaluated in over 400 patients. It was postulated that this could be due to the lack of HLA-DR surface antigens on neonatal fibroblasts, compared to other skin cells which generate the classic allograft rejection (Couno CB et al., 1987).

Clinical studies evaluating the efficacy of treating human diabetic foot ulcers with DG have demonstrated a 50% increase in the healing rates within 12 weeks, and more importantly, a much delayed recurrence of the ulcer in the treated group. It has been suggested that the delay could be due to a more normal healing pattern with DG (Gentzkow GD et al., 1996, Naughton GK et al., 1997)

Tissue engineering therefore offers the ability to replace damaged or destroyed dermis with a manufactured living dermal implant, without increasing patient morbidity.

Dermagraft may be able to 'induce' other indolent ulcers such as venous leg ulcers to heal.

The molecular processes of wound healing and clinical progress to wound closure are the main topics of investigation in this MD thesis.

2 CHAPTER TWO: AIM

This thesis studies the influence of inflammatory cells on human wound healing. After the initial inflammatory response by neutrophils, the majority of the wound inflammatory cells are mononuclear cells. As previously described, macrophages seem to play a key role in the regulation of wound healing, in both degradative and reparative phases of wound repair. Consequently this work concentrates on these cells.

Much of the work into the role of the macrophage in wound healing has been in animals or *in vitro* models. Much of its potential role in human wound healing remains unclear.

Chronic wound healing in humans may be characterised by a reduction of total numbers or activation of wound macrophages, since these cells are essential to the healing process. This thesis aims to morphologically characterise the numbers and macrophage activation over two distinct groups of wounds. Macrophages are studied in chronic non-healing wounds and following two weeks of normal wound healing in PNS wounds.

Attempts to achieve wound closure in recalcitrant wounds have also been conducted in earnest by many industries. If successful these may reduce the yearly health service budgets in the management of non-healing wounds and their associated morbidity, both in the UK and abroad considerably. Recently, a living human dermal replacement, Dermagraft®(DG), has been developed. This aims to be permanently implanted on the wounded tissue to facilitate healing. The hypothesis suggested was that the impaired healing observed in these wound may be due to an abnormal dermis, and by replacing it with a healthy dermis, one could 'switch' these chronic wounds into acute wounds. Should this be the case then one would expect to find changes in the inflammatory profile as healing or wound shrinkage is observed. The aim here is

several-fold, the first and the most important being the safety of the product in patients with venous leg ulcers, the effectiveness of DG (when used in conjunction with multi-layer compression therapy) in the promotion of healing, to determine the optimal treatment application frequency to achieve a clinical benefit, and to elucidate the histological changes taking place within the wound tissue associated with DG treatment.

3 CHAPTER THREE: METHOD

3.1 PILOT STUDY METHOD

3.1.1 Patient group

3.1.1.1 Acute wound healing model

It is common practice at the University Hospital of Wales and Llandough Hospital in Cardiff, that following excision of a pilonidal sinus the wound is left to granulate and heal by secondary intention. This forms an ideal model to observe acute wound healing. Following ethical approval from Bro Taf Ethics Committee and with the permission of all surgeons, patients with such a wound were recruited from surgical units at the University Hospital of Wales, and Llandough Hospital in South Glamorgan. Ten patients with a pilonidal sinus wound were recruited for the study. The patients were seen within a week of the operation and biopsied around 14 days after the excision of the pilonidal sinus.

3.1.1.2 Chronic wound healing model

Patients with venous leg ulcers could be used as a suitable model to observe the chronicity of non-healing wounds. Following ethical approval from Bro Taf Ethics Committee, ten venous leg ulcer patients were recruited.

A detailed medical history was obtained from each patient to identify any relevant past or current factors that could influence their wound healing. Patients below the age of eighteen, pregnancy, co-existing illness, chronic renal failure, known malignancy, or on steroid or immunosupression treatment were excluded from the

study. Full informed consent was obtained from all patients. The patients underwent detailed physical examination and Doppler ultrasound examination of the venous system was also performed to confirm venous leg ulcer aetiology. Those selected demonstrated no progress towards healing despite the use of graduated compression therapy for at least twelve weeks prior to recruitment.

3.1.2 Biopsy Technique

Biopsies were obtained during the second week after the excision of a pilonidal sinus. They were usually taken at 1cm below the wound edge, ensuring true representations of the wound bed. 2mls of 1% Lignocaine was initially injected into the area. A 6mm sterile punch (Stiefel biopsy punch, SFM, Wächtersbach, W.Germany) was then pushed into the tissue with a rotational movement. Once the full length of the blade of the punch had entered the tissue the tissue was 'scooped' out. This technique minimised the tissue damage that may occur when the tissue is handled with forceps. The small amount of bleeding that is usually seen in the tissue bed following biopsy was cauterized with Silver Nitrate sticks and gentle pressure with a gauze swab for a couple of minutes until the bleeding had stopped.

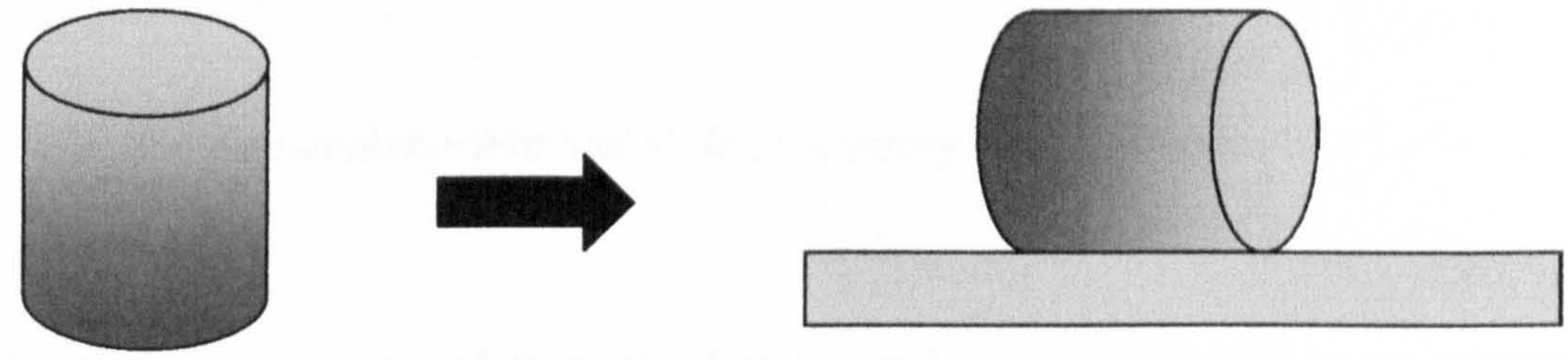
3.1.3 Specimen preparation

Prior to obtaining the biopsy, a skin marker was used to mark the surface of the wound bed lightly, and this mark was incorporated into the biopsy tissue. Although, most biopsies were cylindrical, which aided in the orientation of the tissue during the mounting process, and enabled a longitudinal section through all layers of the wound biopsy could be obtained. Occasionally the biopsy was in the shape of a sphere due the scooping action when using the punch. Here the mark on the surface of the wound aided in the correct orientation of the tissue.

A small amount of Cryo – M Bed (Appendix 7.2) embedding medium was placed on a piece of cork board approximately 10 x 5 millimetre, at room temperature on a small preparation table in the consulting room. The preparation table contained a small beaker of N-Hexane (20 – 30 mls), a pair of forceps, tongs and a dewar flask containing liquid nitrogen.

Within one minute of obtaining the biopsy, it was mounted on its side on the prepared cork board, and the whole specimen was covered with more embedding medium. This ensures a longitudinal representation of the specimen when it is presented for sectioning. (Figure 3-1)

Figure 3-1 Mounting the Specimen



The beaker of N-Hexane was then suspended in a dewar containing liquid nitrogen with a pair of tongs and the prepared specimen on the cork board was immersed into the beaker with a pair of forceps. The specimen was completely immersed in the N-Hexane, whilst it cooled to - 170°C. This was to avoid the formation of ice crystals within the biopsy during the freezing process. The snap freezing of the tissue sample was completed within five minutes of obtaining the specimen. The frozen specimen was then placed in a bijou bottle, labeled, with the patient's initials, date of specimen preparation and stored in liquid nitrogen at -170°C until use.

During sectioning, the specimen was placed on a pre-cooled chuck (-20°C) in a cryostat cutting chamber. The tissue was supported on the chuck by building it up with the embedding medium within the chamber. 6μm sections were cut and mounted onto Poly-L-Lysine (Appendix 7.2) coated microscopy slides. Poly-L-Lysine coated microscope slides enabled the thin tissue sections to be picked up from the platform

adjacent to the cryostat blade with minimal distortion to the architecture of the tissue. The slides were then air dried for about an hour at room temperature, wrapped sequentially in aluminium foil, and stored desiccated at -20°C until processed. The slides were processed within two weeks of cutting.

3.1.4 Immunohistochemical slide processing

The slides were removed from the freezer, and allowed to reach room temperature before the aluminium foil was removed. This prevented condensation on the foil from dissolving any soluble antigens on the sections.

They were then labeled with the patient initials using an engraving pen and were fixed by placing in dried acetone at room temperature for 15 minutes.

Most of the sections cut from the biopsies remained stable and undamaged throughout the various stages of the staining and antibody labelling process.

Excess acetone was removed by air drying the sections for 10 minutes at room temperature. The slides were then washed three times for five minutes in Tris Buffered Saline (TBS) at a pH 7.6. The sections were then incubated with 1% normal bovine serum (ABC Elite Kit, Appendix 7.2) for 20 minutes at room temperature in a humid chamber. (The humid chamber was prepared each morning by soaking paper towels in water and placing them in a tupperware container with a lid, and the slides placed in them for the specific incubation period at room temperature approximately 21 degrees Celsius). This is performed to block any non-specific tissue binding of the antibody and hence reducing the background staining of the sections during subsequent development.

After 20 minutes, the blocking serum was drained off and the sections were incubated with the optimal dilution of the primary antibody for 30 minutes in a humid chamber at room temperature. The slides were washed three times in TBS (five minutes each wash) and incubated with the biotinylated secondary antibody for 30 minutes in a humid chamber at room temperature. Following a further two five minute washes in TBS, the slides were once again incubated in a humid chamber at room temperature with the ABC Complex histochemical staining reagent (Appendix 7.2). This complex is made of avidin - a large glycoprotein extracted from egg white (albumin) and a low molecular weight vitamin called biotin. It is usually prepared at least 30 minutes before incubation to allow the avidin and biotin to react together to form a large labelled complex which can amplify the antigen-antibody reaction in the sections.

The sections were once again washed twice in TBS (five minutes each) and developed with Diaminobenzidine (DAB) substrate (0.005%) (Appendix 7.2) for 10 minutes at room temperature (Appendix 7.1). The slides were rinsed once in TBS and counterstained with Erlichs' Haematoxylin for 30 seconds. The slides were rinsed in tap water and dehydrated by passing through 70% alcohol (Appendix 7.2), 100% alcohol twice, alcohol/xylene mixture, and xylene twice (Appendix 7.2). Five minutes were spent at each dehydration stage. Finally, the sections were mounted under cover slips using DPX (Appendix 7.2).

3.1.5 Monoclonal antibody panel.

The antibodies used and their relevant dilutions are shown in table 3-1.CD68 and 27e10 were available commercially, the MR antibody was a kind gift from P Stahl,

St. Louis, Missouri, USA and the SR antibody was a kind gift from Dr P Crocker, Dundee, UK (Appendix 7.2)

Table 3-1 Panel of Antibodies used in the Pilot Study

Monoclonal Antibody	Target Cell	Dilution
CD68 (monoclonal mouse)	Monocytes, Macrophages	1:50
27e10 (monoclonal mouse)	Early stage Macrophages	1:250
Anti-mannose (MR) (monoclonal mouse)	Primed Macrophages	1:500
Anti-Sialoadhesin (SR) (monoclonal mouse)	Tissue Macrophages	1:20

The final dilutions of the monoclonal antibodies were determined by their ability to bind specifically to the antigen, providing a clear identification of the cells under study without flooding or bleaching the sections.

3.1.6 Analysis

3.1.6.1 Qualitative Evaluation

All sections were examined by both the investigator and an experienced immunologist, Dr Keith Moore (KM) to ensure the quality of the sections were adequate for analysis. Instructions on the identification of the salient features of the different cells were established at the start of the assessment.

The features that helped to identify the different types of cells are summarized below.

Polymorphonuclear neutrophils were cells with multi-lobed dark nuclei and very little cytoplasm.

Macrophages were cells with eccentric 'kidney shaped nuclei, containing granules and vacuoles in the cytoplasm.

Lymphocytes were small round cells with dark round nuclei and very little cytoplasm.

Capillary endothelial cells were large cells with elliptical nuclei encircling vessel-like tubular structures.

Plasma cells were medium sized cells with a large cytoplasm and eccentric oval nuclei, occasionally looking like a 'clock face'.

3.1.6.2 Field Selection

The use of a single section to obtain histological diagnosis is a well-established procedure in routine histopathology. This assumes that the tissue from which this biopsy is obtained, and the subsequent sections are homogenous and thus represents the whole lesion. This may be adequate to generate enough baseline data to allow clinical decisions to be made, recognizing that the lesion may be heterogeneous, e.g. in excluding or verifying neoplastic lesions and lymph node spread.

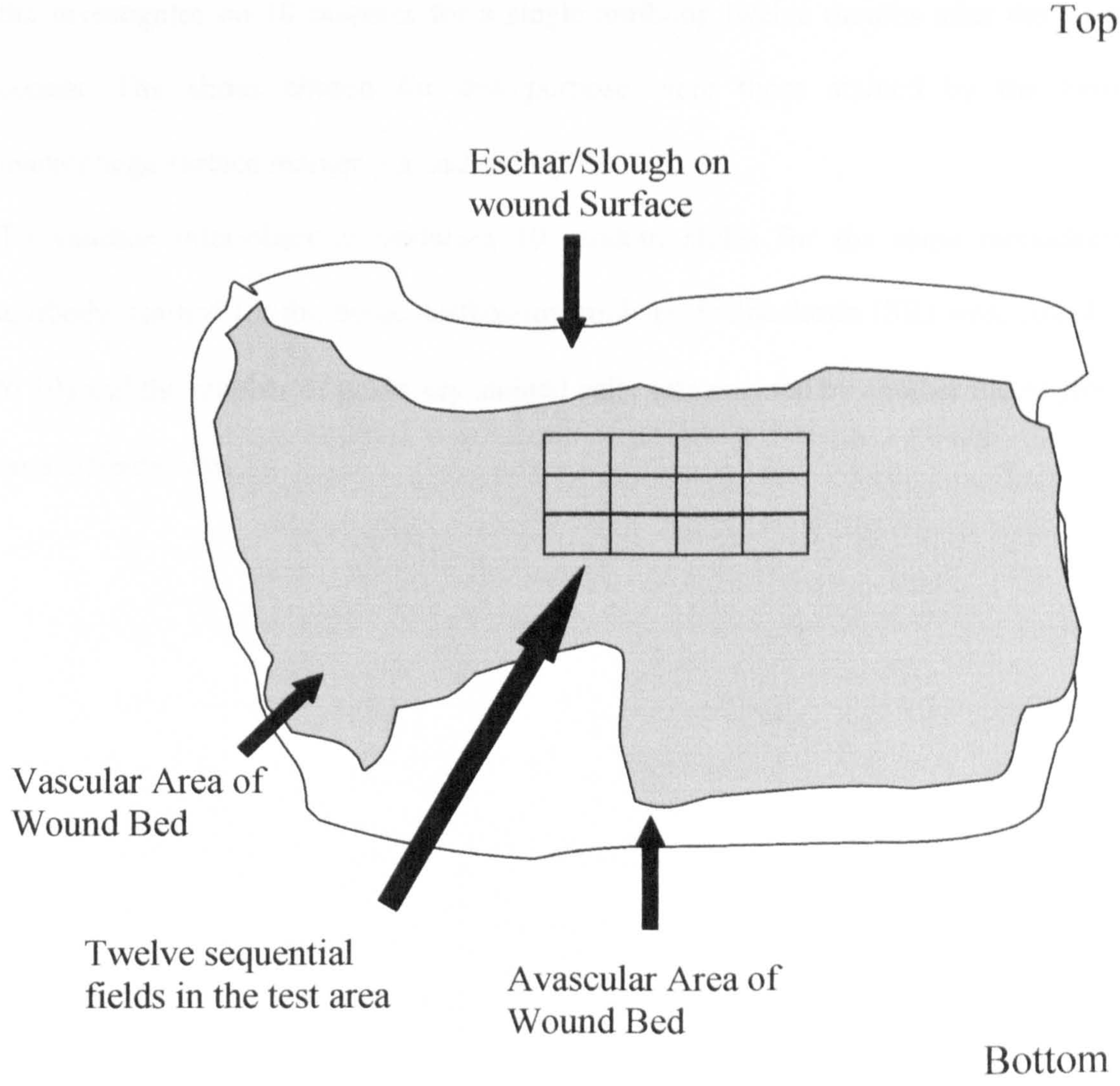
Tissue homogeneity may not apply to acute and chronic leg ulcers, where one area of the lesion may have epithelised and in the case of the chronic wound, another area may be degenerating. Previous work produced from this unit, demonstrated that when a single wound biopsy was completely sectioned and every fifth section stained for a single monoclonal antibody (in this case CD3), the distribution of cells in the vascularised area of a single section were 95% representative to those found throughout the biopsy.

Low power views (x10 magnification) of the wound bed sections demonstrated that the wound had areas of vascularisation with abundant cellular infiltrate and avascular areas with sparse cellular infiltrate.

The macrophage infiltrate of all biopsies was quantified within the vascularised area of the section, by morphological identification and counting the number of positively stained cells at x40 magnification view. This was facilitated by transmitting the microscope image of the section on a visual display screen. To count each positively stained cell without duplication, a computer software programme developed by Dr Peter Plassmann (University of Glamorgan) was used. This highlighted every positive cell counted by a coloured dot. To ensure that the same area is counted for the various

sub-populations of macrophages, the overall shape of the section was documented, an area was randomly selected within the vascular area and this was highlighted in the diagram. This was performed to minimise any errors that may occur by counting different areas for the different antibody positive cells. Having identified the test area the number of positive cells for the different monoclonal antibody was counted in twelve sequential fields as shown in figure 3-2.

Figure 3-2 Field Selection within Wound Bed Section



3.1.6.3 Reproducibility of Counting

To minimise intra-observer bias, only the initials of the patient and the specific monoclonal antibody, prior to examination. The investigator carried out all counting several weeks after the staining process. The type of wound assessed remained unknown until the names of the patients were matched to the wound type at the end of the study.

Intra-observer counting techniques were validated by carrying out duplicate counts by the investigator on 10 biopsies for a single antibody twelve months after the initial counts. The slides chosen for this purpose were those stained by the tissue macrophage surface marker Sialoadhesin (SR).

To validate inter-observer variation 10 random slides for the same monoclonal antibody, stained for the tissue surface macrophage Sialoadhesin (SR) were coded (1 to 10) and the number of positively stained cells was counted by another investigator, on a different day.

3.2 DERMAGRAFT STUDY METHOD

The main body of this thesis consists of a prospective, randomised, open-label feasibility study of 12 weeks duration conducted at 6 centres, 3 in the UK and 3 in Canada. The centres that participated in this study included, Cardiff, Cheltenham, Stockton-on-Tees (Newcastle), Toronto/ Mississauga, Montreal, and Winnipeg. It is estimated that these centres would provide 12 evaluable patients in each treatment arm. The different arms of treatment will be discussed in detail in the following sections.

The primary objective of the study was to assess the safety and effectiveness of DG in conjunction with conventional therapy, in the promotion of healing of venous leg ulcers, compared with the conventional therapy of four layer compression (PROFORE) only.

The secondary objective was to determine the optimal treatment application frequency of DG, with the object of progressing to a pivotal study, comparing conventional four layer compression bandaging (PROFORE) against one treatment application of DG, in conjunction with conventional therapy, with suitable patient numbers.

3.2.1 Patient Population

Sixty three venous leg ulcers patients were screened for eligibility prior to entry into the study at 6 centres. The patient had to fulfill the selection criteria at the screening visit, and give written informed consent to participate prior to study enrolment.

3.2.2 Patient Selection Criteria

All patients entering the study must have the following characteristics:

1. A venous leg ulcer without exposure of muscle, tendon or bone
2. Venous reflux demonstrated by appropriate vascular investigation such as a duplex or a venous Doppler scan of the affected limb, or the patient had a proven history of deep vein thrombosis and/or the appearance of the leg is typical of post-thrombotic limb. Typically this consists of varicosities, a brownish flaky appearance around the malleoli, termed lipodermatosclerosis, and a superficial ulcer.
3. Have an ulcer present for at least 2 months but not more than 60 months prior to screening visit.
4. Have an ulcer between $3cm^2$ and $25\ cm^2$
5. Have an ankle Brachial Pressure Index is ≥ 0.7
6. Patient's ulcer heals less than 50% from the screening visit to the start of treatment (day 0 visit).

Early in the study, it was identified that only 10% of the patients seen in the 6 centres were eligible for study as the target ulcer were usually less $5cm^2$. This was revised to incorporate ulcers of $3cm^2$ or more. The length of time the target ulcer needed to be present to qualify for this study was also amended from 24 months to 5 years, as most of the patients recruited for the study were selected from a sub-population of patients with 'harder to heal' venous leg ulcers.

3.2.3 Ethics

The study protocol, patient information sheet, patient consent form, and information letter to the General Practitioner (GP) were approved by an Institutional Review Body (IRB)/Independent Ethics Committee (IEC) in Canada and the Local Research Ethics Committees (LRECs), in the UK.

3.2.4 Screening and treatment protocol

A screening visit to assess eligibility to enter the study preceded the study visit by a week. All eligible patients were screened for inclusion and exclusion criteria, and signed the informed consent form approved by IRB/IEC or LRECs at the start of the study. The patients' leg ulcer history was recorded, and if the patient had several ulcers, one target venous leg ulcer was chosen and was cleaned according to standard practice at each centre, this ranged from wiping the ulcer with a saline soaked gauze, washing the leg with running water or soaking the study leg in a bucket of water, with or without emollients. If there was evidence of local areas of necrosis or slough within the ulcer bed, they were debrided either surgically or using autolytic agents, at the discretion of the investigator at the centre.

After debridement, the ulcer was photographed and traced onto a transparent ulcer grid measurement sheet, so that its area could be determined. Ulcer tracings and photographs of the wound site were carried out weekly prior to the application of Dermagraft (DG).

Before randomisation, the wound was dressed according to the standard practice of the centre, choosing from a wide range of non-adhesive primary dressings, and then bandaged with a long stretch four-layer bandaging system (PROFORE™)

At the start of the study, (study week 0), the inclusion and exclusion criteria were checked again, in particular the criterion which stated that the ulcer must have not healed by more than 50% following the screening visit.

3.2.5 Selection of DG doses in the study

Previous studies conducted with DG in the treatment of diabetic ulcers, had shown that weekly application of DG for eight weeks resulted in closure of the wound. (Naughton G et al., 1997, Pollack RA et al., 1997)

In this study, three regimens were chosen to encompass the concept that a chronic wound needs a certain amount of stimulation before the process of wound closure will proceed. Once this process has been initiated and a certain rate of wound closure maintained, it is possible that no further treatment will be necessary. Consequently, two extremes of treatment were chosen to determine if a single application of DG at week 0 could impact on venous ulcer healing, or whether the cumulative effect of repeated applications on a weekly basis (weeks 0 – 11 i.e. total of 12 pieces) was important. In addition, a mid-ground dosing regimen was chosen to examine the impact of repeat applications on early stimulation of the wound (weeks 0 and 1), followed by further repeat applications evenly spaced during the course of treatment at weeks 4 and 8, i.e a total of four applications (Weeks 0, 1, 4 and 8).

3.2.6 Patient Treatment Randomisation

Randomisation occurred according to a pre-determined code list for each centre. All patients attended the clinic on a weekly basis even if they did not require DG application. Patients were randomly allocated to one of the four treatment regimens –

Group 1: Patients received one piece of DG from week 0 up to and including week 11, at each weekly visit to give a total of 12 applications

Group 2: Patients received a piece of DG at weeks 0, 1, 4 and 8, a total of 4 applications

Group 3: Patients received only 1 piece of DG at week 0

Group 4: Patients received no DG (Control)

Those patients allocated to the control group had their ulcer covered with a piece of non-adherent Dermanet®, and a four-layer bandage applied. Dermanet is a non-adhesive dressing, similar to many other proprietary dressings available in the community, which is made from an inactive material and hence exhibits no healing properties. Patients receiving DG, had their ulcer covered with DG, a piece of non-adherent Dermanet and four-layer bandaging system.

As previously mentioned, DG was shipped to the various centres in the cryopreserved format at -70°C. Prior to use, DG was thawed to room temperature in a warm water

bath of 37°C for about 3 minutes, rinsed in sterile physiological saline twice for five seconds each, cut to the size of the wound bed, and applied to the wound bed. This was then covered by a piece of Dermanet, and the four layer bandages.

3.2.7 Biopsy Procedure

Biopsies were obtained at week 0 and week 6 of the 12 week study, from the centre of the ulcer using a sterile 6mm-biopsy punch (Stiefel). The wound was infiltrated with 1% plain Lignocaine and the biopsy punch was inserted. The tissue was then 'scooped' out of the wound bed. Haemostasis was achieved by gentle pressure with an absorbent dressing, before redressing the wound as per standard practice. Experience from numerous previous studies has shown this method to be relatively painless.

The first time scale (0weeks) was chosen as a starting point, all patients up until that point having received the same treatment and degree of monitoring. Week 6 was chosen as the point for the second biopsy because previous work from this unit has demonstrated that the maximal immunological and cellular activity, in response to wound manipulation, occurs at this time.

3.2.8 Tissue Preparation

The sample tissue was bisected longitudinally by placing a scalpel over the mid-point of the tissue and pressed firmly with one smooth movement. One half of the biopsy was placed in 10% buffered formal saline and transferred 24 hours later into 50% ethanol for transportation and long-term storage, at room temperature. The other half was snap frozen in liquid nitrogen, as described in section 3.1.3

3.2.9 Assessment of Healing

Photographs and ulcer tracings were carried out weekly in the clinic over the 12 week study period to assess the progression of wound healing. Complete wound closure was defined as full epithelisation of the wound with no drainage. Because initial closure can be difficult to judge due to the thin neoepithelium, complete healing during the primary efficacy period had to be confirmed at the next consecutive visit. Therefore a wound judged to be healed at one visit but open at the next was considered to have not healed.

Although weekly photographs of the target ulcer were taken, for the duration of the study period, some of the photographs were of poor quality, being too dark or overexposed, making the assessment of the progression to wound closure difficult. Ulcer tracings were however a more reproducible way of assessing progression.

In those wounds that remained unhealed at the end of the study period, the area of reduction in the ulcer size was expressed as a percentage for ease of comparison between the groups, this was calculated as:-

Equation 1. Percentage Reduction in Ulcer Size

$$\text{Percentage Reduction} = \frac{\text{Week 0 area} - \text{Final area}}{\text{Week 0 area}} \times 100$$

Any adverse events during the study period were documented.

The wound was said to be infected if it demonstrated at least two of the following:

Abnormal purplish granulation tissue with contact bleeding,

Exudate

Pain

Superficial Bridging

Smell

Lack of progress to healing as documented by an increase in the size of a wound

This criteria of wound infection classification was first described by Cutting and Harding (Cutting K and Harding KG, 1994)

3.2.10 Histology

Each fixed biopsy was placed in a capsule and passed through seven serial concentrations of alcohol and xylene. The specimens were then carefully embedded in paraffin wax and orientated so that sections could be cut vertically through the thickness of the wound biopsy. After solidification, 5μm sections were cut and mounted onto poly-L-lysine coated slides and left overnight to dry at 60°C in an oven. The sections on the slides were then rehydrated by passing the slides through xylene for 5 minutes, followed by two passes through 100% alcohol for 3 minutes. They were then stained with Haematoxylin and Eosin (H&E).

The other half of the biopsy that was snap frozen was cut into 6μm thick sections using a microtome and mounted on poly-L-lysine coated slides.

Using the immunohistochemical processing method previously described in 3.1.4, sequential sections were stained by a panel of monoclonal antibodies to demonstrate the various inflammatory cell types involved in wound healing. The antibodies used, their relevant dilutions, and the order of staining is shown in Table 3-2 and Appendix 7.2. The order of staining was important because it ensured that the macrophage sub-populations, the lymphocytes, and cytokines were on adjacent sequential sections, and thus aided in the subsequent immunohistochemical analysis of the tissues. To minimise any aberrant antigen-antibody reaction and staining patterns due to environmental factors such as the humidity and ambient temperature of the laboratory, week 0 and week 6 sections for one patient were processed in one sitting.

Neutrophils, which are the first inflammatory cells to be encountered in wounded tissue were stained in further cut wax sections. Cryostat sections were abandoned for this antibody because the antigen diffused out of the cells and the cells were surrounded by a 'cloud' of labeling. Wax sections however yielded specific cellular staining. The wax sections were brought to water as described above, and stained for neutrophils using the Avidin-Biotin peroxidase technique, as previously described, and developed with DAB substrate.

Table 3-2 Antibody Panel used in the Assessment of Frozen sections in the Main Study (Appendix 7.2)

Monoclonal Antibody	**Target Cell**	**Dilution**
TNFα (mouse)	Macrophage Cytokine	Neat
IL1β(mouse)	Macrophage Cytokine	1:25
CD68 (mouse)	All Monocytes and Macrophages	1:50
27e10 (mouse)	Early stage Macrophages	1:250
Mannose (mouse)	Primed Macrophages	1:500
Sialoadhesin (mouse)	Tissue Macrophages	1:20
CD3 (mouse)	All T Lymphocytes	1:50
CD4(mouse)	T_{helper} Lymphocytes	1:10
CD8 (mouse)	$T_{suppressor}$ Lymphocytes	1:30
CD19(mouse)	B Lymphocytes	1:25
CD45 (mouse)	All Leucocytes`	1:20
Von Williebrand Antigen (rabbit)	Factor VIII related antigen on Endothelial cells	1:150
VEGF (mouse)	Endothelial Cytokine	1:100
bFGF (mouse)	Macrophage Cytokine	1:150
TGFβ (rat)	Macrophage Cytokine	1:400
Neutrophil Elastase (mouse)	Neutrophils	1:200

3.2.11 Analysis

3.2.11.1 Clinical

The primary end point in the clinical assessment was complete wound closure by week 12. The secondary end point was to assess the reduction in the ulcer area at the end of the 12 week study even if complete closure was not achieved. Finally, and most importantly, the safety of DG was assessed by documenting all the adverse events that occurred to the study patients during their trial period, even when the adverse event was not directly related to the application of DG.

3.2.11.2 Histological

3.2.11.2.1 *Wax sections*

Each patient was assigned a random number. Week 0 and week 6 biopsies were randomly assigned letters a and b. The sections were analysed as a pair by two assessors who were blinded as to which biopsy was week 0 and which was week 6.

Both assessors analysed the sections for the following

1. Thickness of granulation tissue (μm)
2. Pattern of collagen and the distribution of extracellular matrix
3. Blood vessel volume and distribution
4. Percentage of blood vessel cuffing
5. Cell profile – polymorphonuclear cell levels, lymphocyte cell levels
6. The degree of fibrosis at the base of the sections

Histological examination of the changes between week 0 and 6 biopsy sections concentrated on three areas

1. The extracellular matrix and collagen distribution
2. The nature and distribution of the blood vessels
3. The types of cells present in the wound tissue.

Extracellular matrix and collagen distribution

The measurements were mostly semi-quantitative, but as the biopsies were assessed in pairs, **a** and **b,** the findings were sufficiently robust to detect any gross differences. The amount of extracellular matrix present was graded as none, low, medium, high or full and its distribution was evaluated as patchy or even on photomicrograph images captured using Photonics Science digital camera and Image Proplus 4 software.

The thickness of the granulation tissue was measured from the junction between the exudate and granulation tissue to the junction of the granulation tissue and the underlying scar or dermis using an eye-piece with a micrometer.

The pattern of the collagen 1mm from the wound surface was classified as broad mature bundles, intermediate bundles, fine fibres, or purely myxoid.

The degree of fibrosis at the base of the biopsy specimens was semi-quantatively graded as Normal (basket weave pattern composed of normal thickness fibres), Medium (orientated fibres composed of normal thickness fibres), and Dense (orientated fibres composed of densely packed fine collagen)

The nature and distribution of the blood vessels

The number of vascular units seen per mm^2 was counted in all sections. These were usually arranged in lobules as clusters of proliferating capiillaries similar to an angioma that is being fed by a vessel from the deep dermis.

The orientation of the vessels to the surface of the biopsy was also recorded, and this was classified as random or perpendicular to the wound surface. The percentage of vessels with fibrin cuffs in the granulation tissue were estimated by observing the degree of cuffing and described as none, medium or high. From this the approximate percentage of blood vessels in the ulcer with any degree of fibrin cuffing was calculated.

Cellular pattern and distribution

The type and distribution of the cells present were evaluated. The cellular infiltration within the granulation tissue was assessed separately for the polymorphs and the lymphocytes, using a grading scale

1. No significant infiltrate - 0%
2. Intermediate between 1 and 3
3. Occasional aggregates - 25 %
4. Intermediate between 3 and 5
5. Several aggregates or moderately diffuse infiltration - 75%
6. Intermediate between 5 and 7
7. Confluent aggregates – 100%

Values 2, 4 and 6 were usually used for adjustment when the two paired biopsies were compared directly

3.2.11.2.2 *Frozen sections*

Field Selection

Low power views of the tissue quickly demonstrated that the sections were made up vascular and avascular areas. As in the pilot study, the overall shape of the section was documented and the area counted was highlighted in the in the diagram. This was performed to ensure that the same area was counted for the various inflammatory cells, thus minimising any errors by counting different areas for different cells. Once

the test area was identified, the number of positive cells was counted in 12 sequential fields.

The inflammatory cells were quantified by morphological identification and counting at x40 magnification. This was performed as before by projecting the microscopic image onto a computer screen and each positively stained cell was counted without duplication using the software programme as in the pilot study.

The number of blood vessels was counted for the whole section, and was standardised by representing the data as the number of blood vessels per unit area.

Cytokine expression within the wound bed sections were graded on a 1 to 4 basis, where

1 = 0 – 24% of the cells positive

2 = 25 – 49% of the cells positive

3 = 50 – 74% of the cells positive

4 = 75 – 100% of the cells positive.

3.2.12 Statistical Analysis

3.2.12.1 Statistical analysis of clinical outcomes in the DG study

The primary endpoint in this study was complete healing. This study was not powered to detect a difference in the proportion of patients who healed by week 12, but it was planned to conduct pair wise comparisons as descriptive summary statistics. Fisher's exact test was used for the pair wise comparisons as the sample size and numbers healed were low.

The secondary efficacy endpoint was time to wound closure and this was represented as the median closure time. No formal hypothesis testing was conducted owing to the low number of patient healing in each treatment group.

3.2.12.2 Statistical analysis of wax sections in the DG study

The relationships of the treatment regimen to histological changes were examined. For each of the DG treatment groups compared with control, any changes from week 0 to week 6 in histology measures were assessed. Fisher's exact test was applied to the binary histology measures at week 0 and week 6 (i.e. two by two tables) and the Mann-Whitney U test was used for all other comparisons.

3.2.12.3 Statistical analysis of the frozen sections in the Pilot and DG study

The cell type distributions in the sections for the pilot study and the DG study were compared using the Mann-Whitney U test.

Differences in the cytokines for the different treatment times and groups were compared using a simple comparative analysis based on an increase or decrease in the intensity of the staining.

4 CHAPTER FOUR: RESULTS

4.1 PILOT STUDY

4.1.1 Patient group

Ten patients with clinically non-infected pilonidal sinus wounds and ten patients with clinically non-infected venous leg ulcers were recruited into the pilot study. All twenty patients matched the inclusion criteria outlined in section **3.1.1.** Patient compliance was 100% for this part of the study.

4.1.2 Biopsies

All biopsies were easily obtained with minimal discomfort to the patient following infiltration of 1% plain Lignocaine into the wound bed. The use of a 6mm punch resulted in a good sample yield. The biopsy was robust and withstood handling during the freezing process. Although minimal bleeding was anticipated and encountered in most patients of both groups, two patients in the venous leg ulcer group needed prolonged compression and a review of the wound two days after the biopsy. In these patients, the biopsy was carried out near a blood vessel, which was partially transected in the process. However there were no long-term sequelae as a result of this. Therefore a total of 20 biopsies were available for the pilot study.

4.1.3 Specimen preparation

Microtome sections of 6μm in thickness were found to give the most stable slice with minimal distortion and disintegration of the tissue whilst cutting and mounting onto poly-L-lysine coated slides.

4.1.4 Analysis

4.1.4.1 Microscopic Analysis

Identification of the inflammatory cell groups was accomplished morphologically, and by staining for expression of subset-specific antigens. Positively stained cells were brown in colour and the negatively stained cells were blue. Cell populations were quantitatively assessed using the counting methods described in section 3.1.6.2

4.1.4.2 Qualitative Evaluation

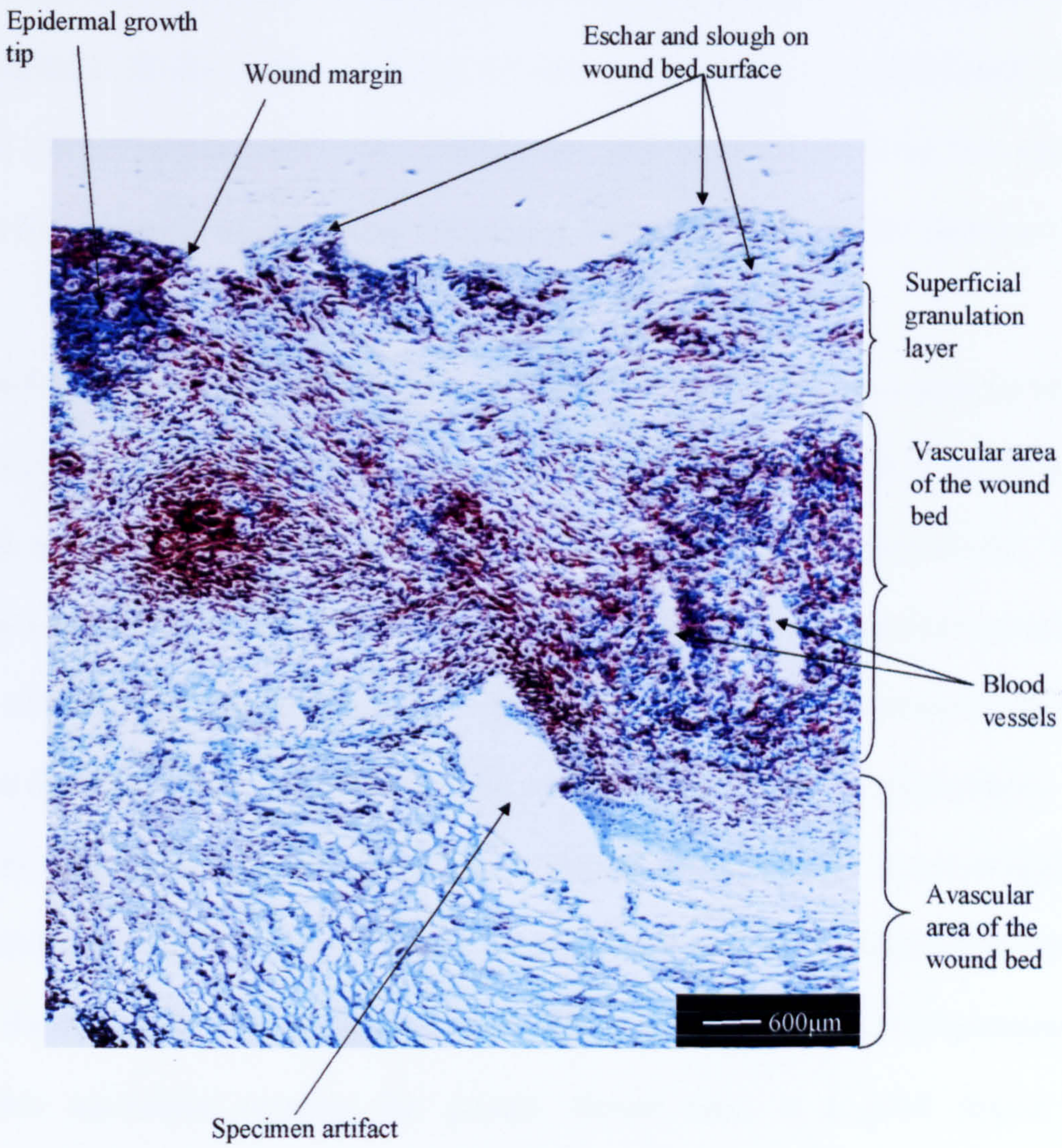
The salient features of individual cells in addition to those cells positively stained by DAB were identified and the criteria for assessing the sections were established as previously described in section 3.1.6.1.

4.1.4.3 Field selection

Low power views (x10 magnification) of the wound bed sections quickly demonstrated that the wound had areas of vascularisation with abundant cellular infiltrate and avascular areas with sparse cellular infiltrate.

The macrophage infiltrate of all biopsies was quantified within the vascularised area of the section, by morphological identification and counting the number of positively stained cells at x40 magnification view. The overall shape of the section was documented to minimise any errors that may occur by counting different areas for the different antibody positive cells. (Figure 4-1)

Figure 4-1 Low power photomicrograph depicting the salient features of a wound bed biopsy



The vascular area contained the abundant cells and the avascular area was predominantly made up of connective tissue with little cellular infiltrate.

4.1.4.4 Reproducibility of counting

A more useful indication of the reproducibility of counts is the standard deviation of the differences between the first and second counts, and between investigator 1 and investigator 2. This allows for the assessment of the differences between the counts, whilst excluding differences between separate biopsies. By comparing this figure with the magnitude of the counts, a degree of reproducibility can be appreciated. This method can be further verified by plotting the difference between the two counts against the mean, as described by Bland and Altman (Bland JM and Altman DG, 1986).

Table 4-1 summarises the mean positive cell/field of 12 fields of two separate blind counts by the investigator for the monoclonal antibody SR stained cells. It can be seen that the mean values for the two counts were 34.0 and 33.5 respectively. The Pearson's correlation coefficient for this set of data was 0.97. The standard deviation of the differences between the counts was 4.2, which was small compared to the standard deviation of the counts themselves, which were 10.4 and 9.6 respectively.

When the differences between the two counts for SR^{+ve} macrophages is plotted against the mean of both counts (Figure 4-2), the counts displayed a pattern distributed evenly around the 0 level. 80% of the counts were within ±5 of the differences demonstrating reasonable agreement between the groups. Hence there is a good degree of reproducibility between the two counts.

Table 4-1 Results of two separate blind counts of SR monoclonal antibody stain in ten chronic wound sections

Patient No	1st Count (SD)	2nd Count (SD)	Mean	Difference
1	10.3 (5.3)	17.5 (8.4)	13.9	-7.2
2	39.7 (11.5)	37.1 (8.5)	38.4	2.6
3	27.7 (7.6)	25.4 (7.4)	26.6	2.3
4	63.3 (13.8)	60.9 (12.8)	62.1	2.4
5	78.9 (23.4)	64 (20.4)	71.5	14.9
6	34.4 (14)	41.7 (10.7)	38.1	-7.3
7	23.9 (7.6)	27 (7.8)	25.5	-3.1
8	4.3 (2.9)	4.5 (3.9)	4.4	-0.2
9	34 (14)	37.6 (10.9)	35.8	-3.6
10	23.2 (4.1)	18.9 (5)	21.1	4.3
MEAN	**34.0(10.4)**	**33.5 (9.6)**	**SD**	**4.2**

Figure 4-2 Differences between two separate blind counts of SR^{+ve} macrophages plotted against their mean demonstrating reasonable agreement between the groups

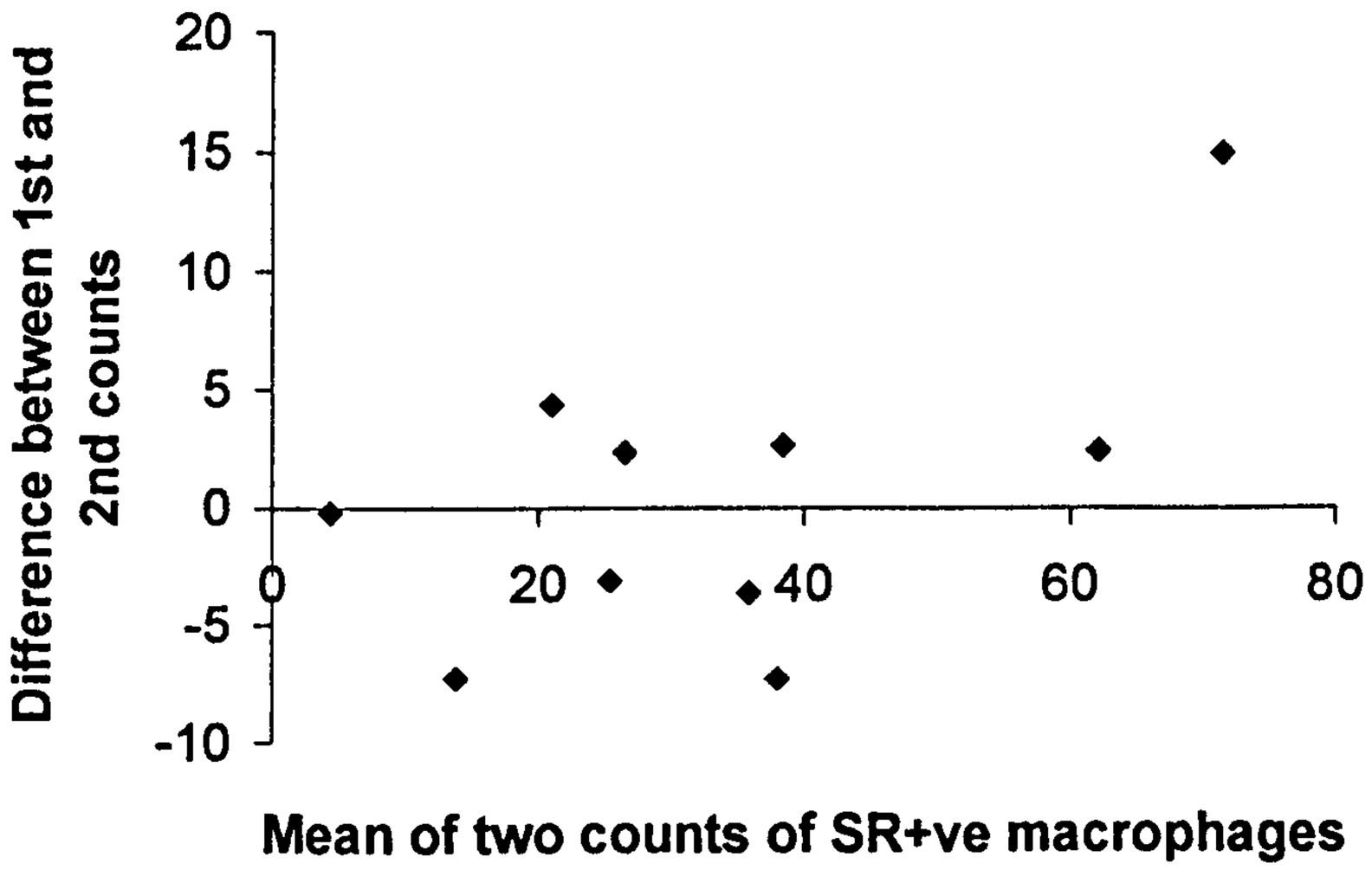


Table 4-2 summarises the mean counts two blind counts by two investigators of the SR monoclonal antibody stain for five acute and five chronic wound sections.

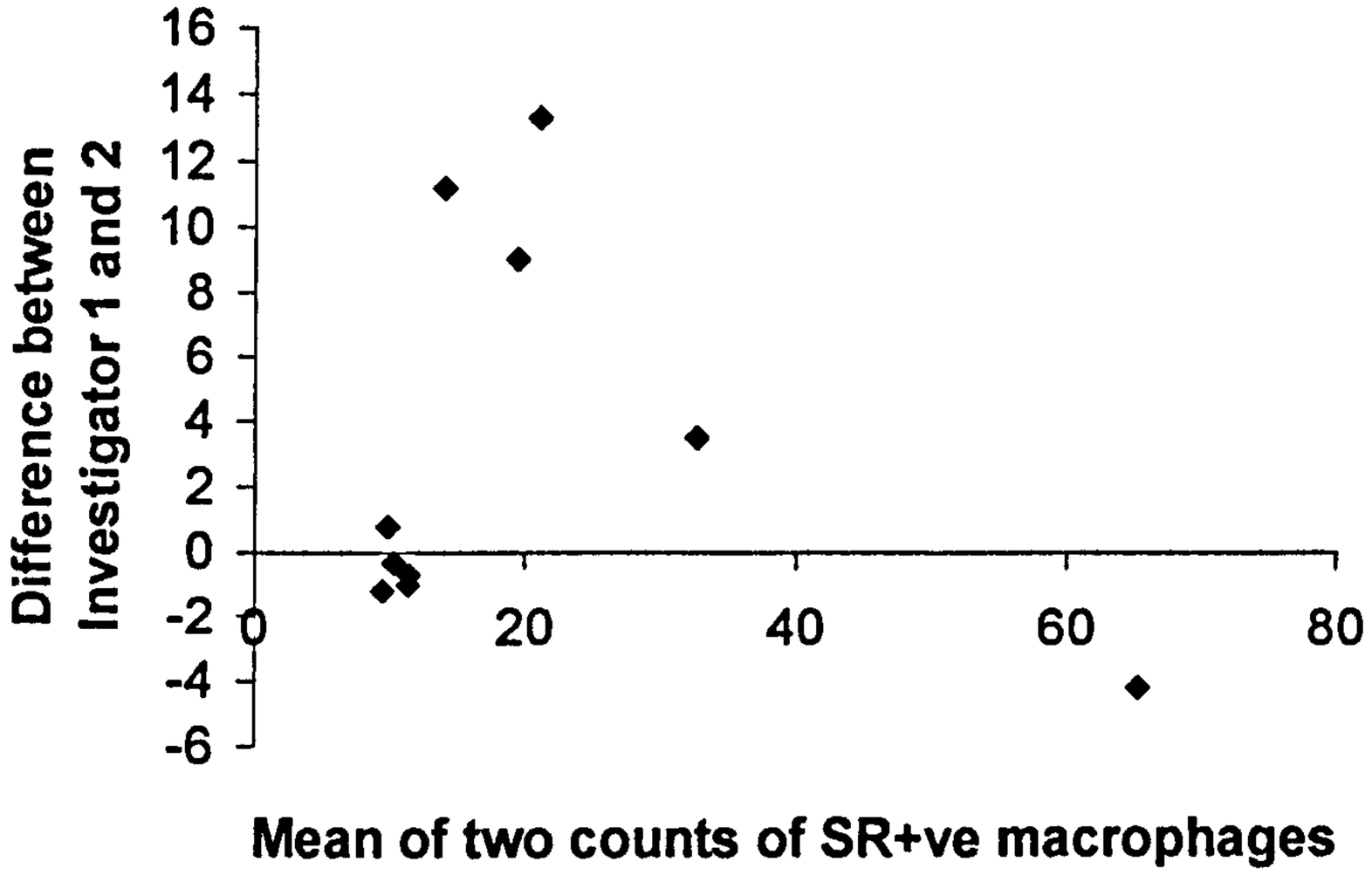
The mean for the first investigator was 22 and for the second was 19. The Pearson's correlation coefficient for the investigators is 0.94. The standard deviation of the difference between the counts was 4.9, this value is smaller when compared to the standard deviation of the two counts themselves, which were 7.1 and 8.8 respectively. Figure 4-3 is a plot of the differences between the counts of two investigators of SR^{+ve} macrophages. For lower mean values the differences are small, but for the larger means, there tended to be a splay. There was also a tendency for investigator 1 to have

higher counts for the larger means. However, it can be shown that there seems to be an acceptable degree of reproducibility between the two investigators.

Table 4-2 Results of two separate blind counts by two investigators of SR monoclonal antibody stain

Patient No	1st Investigator Mean (SD)	2nd Investigator Mean (SD)	Mean	Difference
1	10.3 (4.5)	10.6 (8.1)	10.4	-0.3
2	8.9 (3.9)	10.1 (5.2)	9.5	-1.2
3	10.9 (3.2)	11.9 (10)	11.4	-1
4	19.6 (9.0)	8.4 (5.5)	14	11.2
5	11.1 (3.0)	11.8 (10.3)	11.5	-0.7
6	10.3 (5.3)	9.5 (4.6)	9.9	0.8
7	23.9 (7.6)	14.9 (5.9)	19.4	9
8	27.7 (7.6)	14.4 (6.7)	21.1	13.3
9	63.3 (13.8)	67.5 (18.2)	65.4	-4.2
10	34.4 (14.0)	30.9 (13.8)	32.7	3.5
MEAN	**22.0 (7.1)**	**19.0 (8.8)**	**SD**	**4.9**

Figure 4-3 Difference between two different investigator counts of SR^{+ve} macrophages plotted against their mean demonstrating an acceptable degree of reproducibility between the two investigators



In summary, it was possible to demonstrate reproducibility of the counts by the investigator on two separate occasions, and correlation in the counts between investigators 1 and 2.

4.1.4.5 Macrophage sub-population distribution in acute and chronic wounds

4.1.4.5.1 *Pan Macrophage Marker CD68*

Both wound types demonstrated an abundance of monocytes and macrophages within the sectioned biopsy from the wound bed, stained by the pan-macrophage surface antigen CD68. The maximal concentration of macrophages could be seen around the blood vessels. As previously stated, due to the abundance of macrophages identified by the CD68 antibody, it was impossible to obtain a numerical count of this antigen. Hence a scoring system was introduced to standardise the evaluation of these slides. A score of grade 3 denotes 50 – 74% of all cells within the section staining positive for the CD68 antigen, and a score of grade 4 denotes 75 – 100% of all cells staining positive for the CD68 antigen. (Table. 4-3)

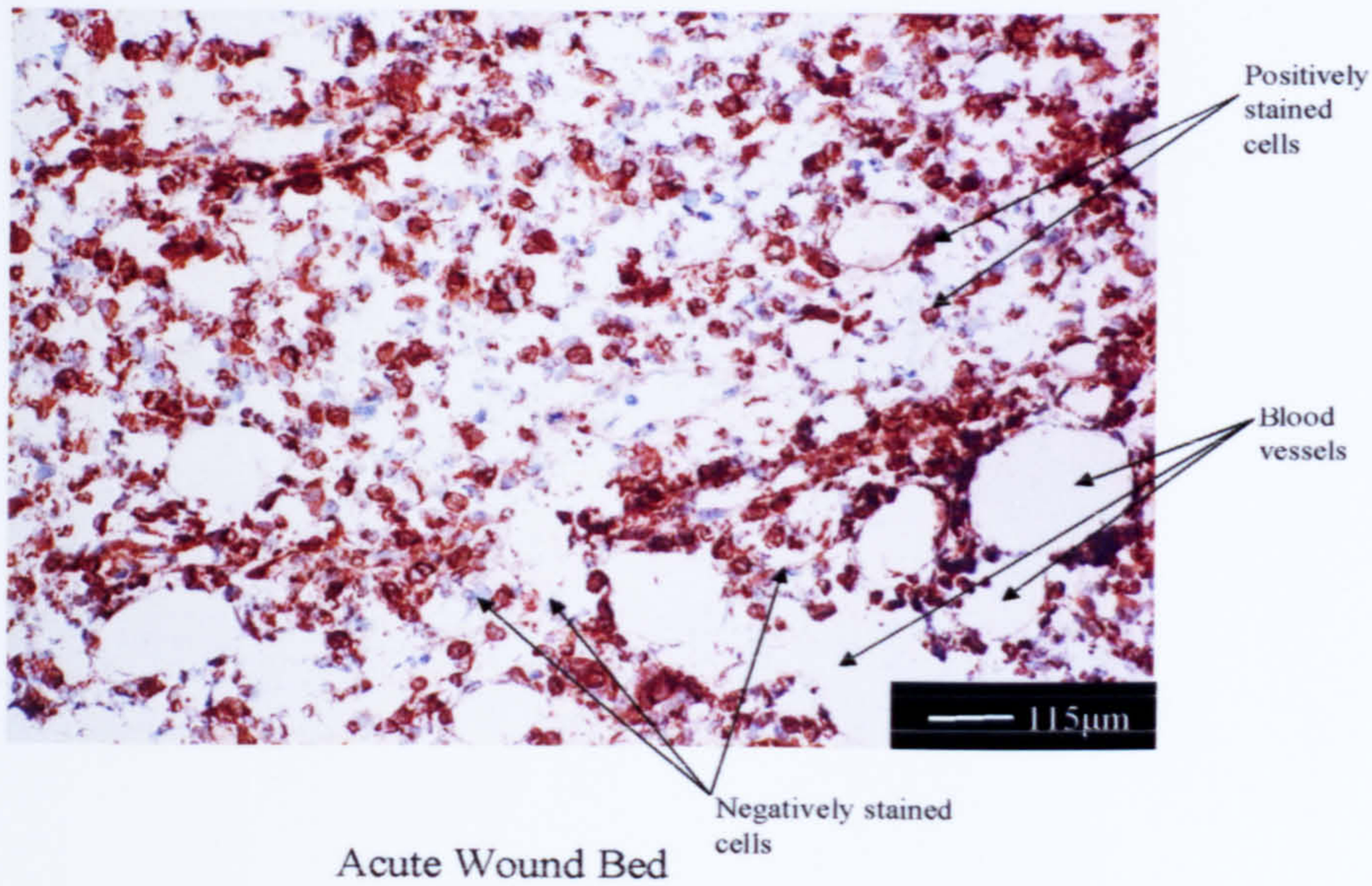
For the other macrophage sub-population markers the mean count of positively stained cells (and the standard deviation) was calculated.

Table 4-3 Distribution of the Pan-Macrophage MarkerCD68 in Acute and Chronic Wounds (Grades)

Patient No.	Acute Wounds	Chronic Wounds
1	3	4
2	4	3
3	4	4
4	3	3
5	4	4
6	4	4
7	4	4
8	3	3
9	3	3
10	3	4

From the above table it can be seen there is an abundance of monocytes and macrophages within the acute and chronic wound beds. This suggests that both healing and non-healing wounds have large numbers of macrophages and given the right condition all wounds should heal. The photomicrographs of the two wound types also exhibit these observations. (Figure.4-4)

Figure 4-4 Distribution of $CD68^{+ve}$ Cells in the Beds of Acute and Chronic Wounds



This shows that both healing and non-healing wounds have large numbers of macrophages

4.1.4.5.2 *Early Macrophage Marker – 27e10*

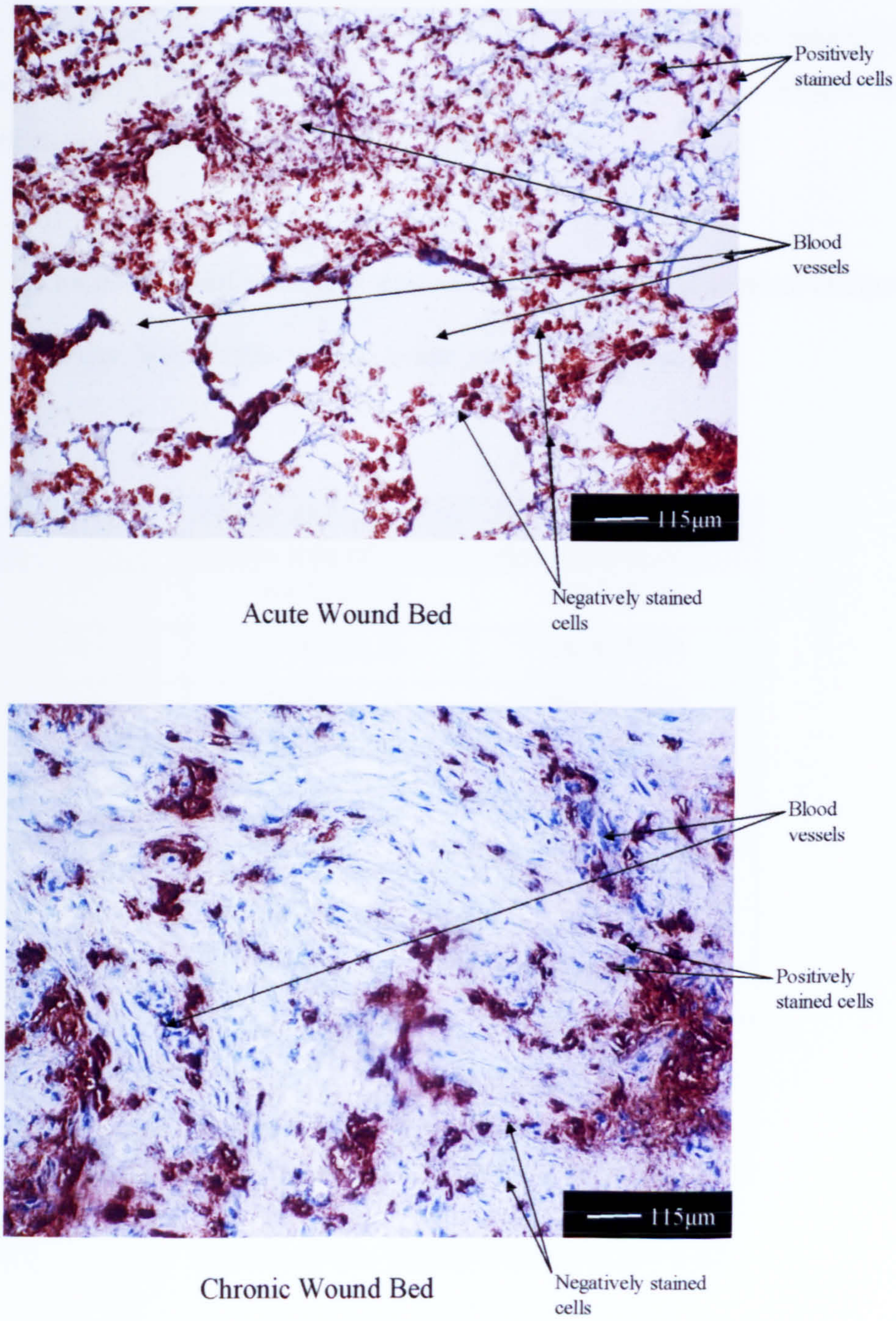
The mean count of the early stage macrophages within the acute wound is 20.8 (8.6) and within the chronic wound is 10.4 (6.7). These are significantly different ($p<0.01$).(Table. 4-4) The higher mean count of $27e10^{+ve}$ cells within the acute wound when compared to the chronic wound can also be seen in (Figure 4-5)

Table 4-4 The Mean Distribution (and Standard Deviation) of Early Macrophages $27e10^{+ve}$ Cells Within Acute and Chronic Wounds

Patient No.	Mean (SD) cells in acute wounds	Mean (SD) cells in chronic wounds
1	16.1 (10.4)	9.5 (3.8)
2	19.1 (7.9)	10.4 (3.1)
3	22.2 (4.3)	13.0 (6.6)
4	27.1 (11.7)	13.2 (16.9)
5	21.7 (5.7)	4.7 (4.3)
6	40.3 (20.4)	5.9 (4.4)
7	17.9 (5.3)	11.3 (11.2)
8	21.3 (8.2)	25.9 (9.3)
9	29.0 (7.4)	4.2 (3.0)
10	14.6 (4.8)	6.3 (5.1)
Mean (SD)	**20.8 (8.6)**	**10.4 (6.7)**

Statistical Test: Mann-Whitney U $p \leq 0.01$

Figure 4-5 Distribution of $27e10^{+ve}$ Cells in the Beds of Acute and Chronic Wounds



This shows that there are more $27e10^{+ve}$ cells in acute wounds than in chronic wounds

4.1.4.5.3 *Primed Macrophage Marker – MR*

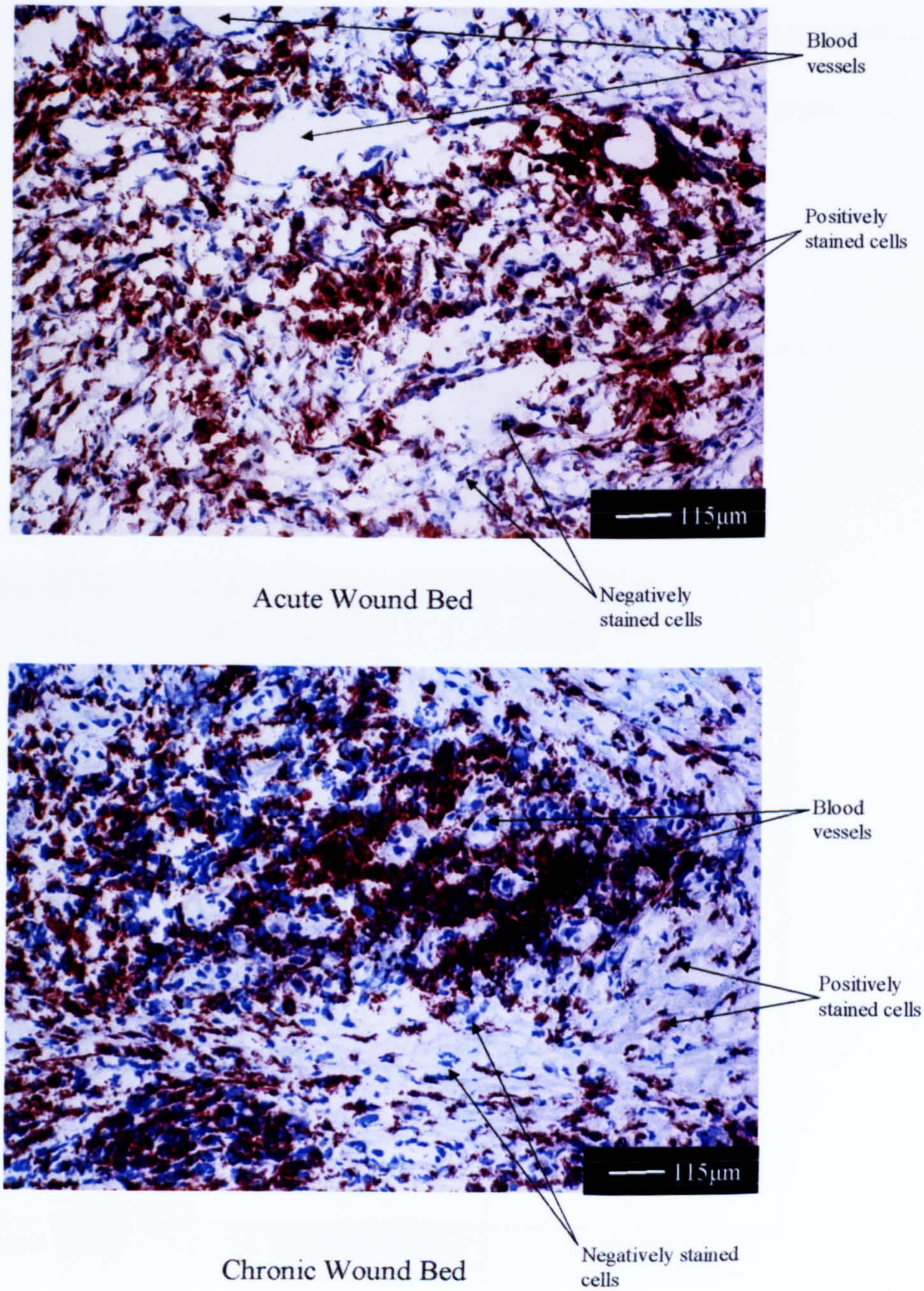
The mean count of primed macrophages within the acute wound was 55.4 (15.2). This was similar to the mean count seen in the chronic wound of 54.3 (19.2) and was not statistically significant (p=0.82). This implies that the distributions of primed macrophages within the acute and chronic wound types were of similar magnitude. This suggests that until these macrophages are activated both wounds behave in a similar manner in response to injury. (Table 4-5)(Figure 4-6)

Table 4-5 The mean (and standard deviation) of the primed surface antigen marker MR^{+ve} cells within acute and chronic wounds

Patient No.	Mean (SD) cells in acute wound	Mean (SD) cells in chronic wounds
1	64.2 (19.8)	28.7 (7.1)
2	54.8 (13.3)	68.8 (20.9)
3	78.3 (23.7)	50.3 (22.7)
4	36.1 (8.4)	76 (32.9)
5	59.2 (12.8)	82.2 (37.3)
6	43.7 (13.6)	67.7 (19.8)
7	72.1 (20.2)	45.3 (17.2)
8	50.7 (11.0)	32.2 (10.0)
9	47.6 (15.8)	53.3 (10.4)
10	47.7 (13.4)	39.0 (13.7)
Mean (SD)	**55.4 (15.2)**	**54.3 (19.2)**

Statistical test Mann-Whitney U p = 0.82

Figure 4-6 The Distribution of MR^{+ve} Cells in the Beds of Acute and Chronic Wounds



The distributions of primed macrophages within the acute and chronic wound types were of similar magnitude

4.1.4.5.4 *Tissue Macrophage Marker - SR*

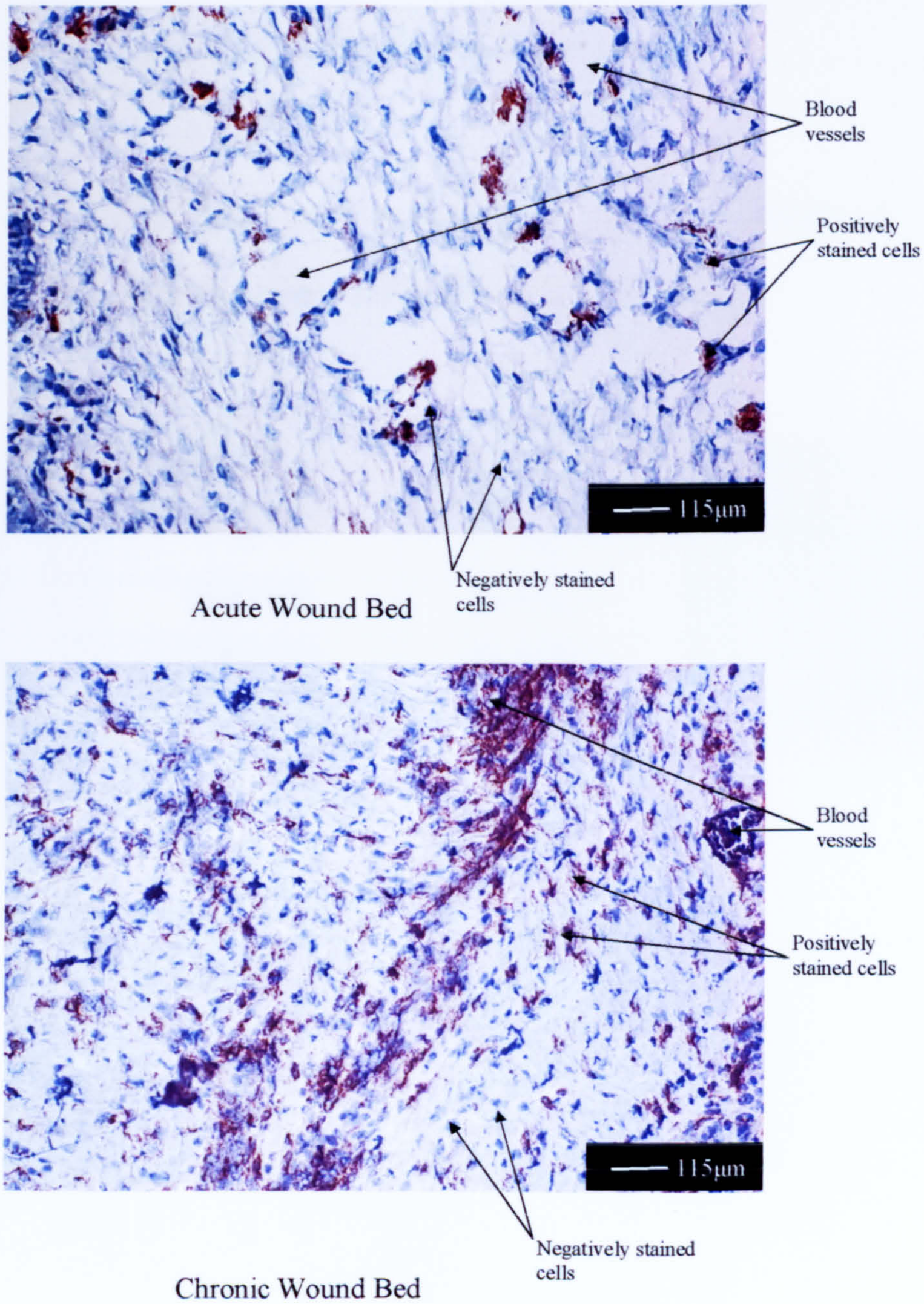
The mean count of the tissue macrophage marker SR^{+ve} cells for the acute wound was 10.9 (4.4) and for the chronic wound was 34.0 (10.5), which was statistically significant ($p<0.01$). (Table 4-6) This suggests that there were a greater proportion of tissue macrophages within the chronic wounds, when compared to acute wounds. (Figure 4-7)

Table 4-6 The mean distribution (standard deviation) of tissue macrophages SR^{+ve} cells within acute and chronic wounds

Patient No.	Mean (SD) cells in acute wounds	Mean (SD) cells in chronic wounds
1	8.9 (3.9)	10.3 (5.3)
2	10.2 (4.5)	39.7 (11.5)
3	7.7 (4.7)	27.7 (7.6)
4	6.8 (2.3)	63.3 (13.8)
5	9.0 (4.0)	78.9 (23.4)
6	19.6 ((9.0)	34.4 (14.0)
7	14.8 (5.8)	23.9 (7.6)
8	11.1 (3.0)	4.3 (4.1)
9	10.9 (3.2)	23.2 (4.1)
10	9.9 (3.1)	34.0 (14.0)
Mean (SD)	**10.9 (4.4)**	**34.0 (10.5)**

Statistical TestMann-Whitney U $p<0.01$

Figure 4-7 The Distribution of SR^{+ve} Cells in the Beds of Acute and Chronic Wounds



Acute Wound Bed

Chronic Wound Bed

This suggests that there were a greater proportion of tissue macrophages within the chronic wounds, when compared to acute wounds

In summary, both acute and chronic wounds exhibit similar magnitude of the pan-macrophage marker $CD68^{+ve}$ cells and the primed macrophage marker MR^{+ve} cells. However, acute wounds demonstrate a significantly larger mean count of the early macrophage marker $27e10^{+ve}$ cells than chronic wounds, and chronic wounds demonstrate a significantly larger mean count of the tissue macrophage marker SR^{+ve} cells than the acute wounds. This is summarized in Table 4-7.

Table 4-7 Cumulative mean distribution of the sub-population of macrophage within acute and chronic wounds.

Macrophage Antigens	Mean (SD) cells in acute wounds	Mean (SD) cells in chronic wounds	Stastistical significance
CD68	4	4	No
27e10	20.8 (8.6)	10.4 (6.7)	Yes
MR	55.4 (15.2)	54.3 (19.2)	No
SR	10.9 (4.4)	34 (10.5)	Yes

4.2 DERMAGRAFT STUDY

4.2.1 Patient population

The study involved 6 centres, 3 centres in the UK and 3 centres in Canada and was live for 8 months from the time the first patient entering the study to the completion of the last patient.

63 patients were assessed at the screening visit. After one or two weeks of multi-layer compression therapy prior to week 0, 10 patients were not randomised at the week 0 visit. The reasons for this is summarised in Table.4-8

Table 4-8 Summary of patients not randomised

Centre	**Sex**	**Age**	***Weeks of Compression***	***Reason for withdrawal***
Cardiff	M	70	1	Ulcer almost healed
Cardiff	F	79	2	Ulcer infected
Cardiff	M	74	1	Ulcer healed 66%
Cheltenham	M	85	1	Withdrew consent
Cheltenham	M	72	1	Withdrew consent
Cheltenham	F	84	1	Withdrew consent
Toronto	M	81	2	Ulcer infected
Toronto	F	73	2	Ulcer infected
Mississauga	F	67	2	Ulcer infected
Winnipeg	F	75	1	Ulcer healed

4.2.2 Ethics

Original and amended protocols were assessed and approved by the two ethical bodies with minimal disruption to the study, the Independent Ethics Committee (IEC) in Canada, and Local Research Ethics Committees (LRECs) in the UK.

4.2.3 Screening and treatment protocol

Out of the 63 patients screened, 53 patients entered the randomisation phase of the study. There were 22 men and 31 women, with an overall mean age of 68.7 years (range 22 - 90 years). The overall mean age for the 22 men was 68.7 (range 22 – 83 years) and for the women was 69.7 (range 37 – 90 years). The overall mean body weight of these 53 patients was 87.3 Kg (range 50 – 155 Kg), men 94.1 Kg (range 50 – 155 Kg) and women 79.3 Kg (range 50.9 – 143 Kg) and the body mass index was 30.4 (range 19 – 61.9) men 30.6 (range 19 – 47 8) and women 30.4 (range 19.3 – 61.9). The mean ankle-brachial pressure index was calculated to be 1.1 (range 0.7 – 1.6), men 1.07 (range 0.7 – 1.6) and women 1.1 (range 0.7 – 1.5).

Out of the 53 patients who eventually enrolled into the study, superficial venous reflux was demonstrated in 45 out of 49 patients and deep vein reflux was confirmed in 44 out of 49 patients. Reflux could not be assessed in 4 patients due to non-availability of hospital ultrasound equipment. These patients however were clearly found to have clinical evidence of venous ulceration.

The demographics were evenly distributed across the four randomisation groups. (Table 4-9)

Table 4-9 Demographics of patients across the four treatment groups

Group	Number		Mean Body Weight (Kg)		Mean Body Mass Index		Mean Ankle Brachial Pressure Index	
	Men	Women	Men	Women	Men	Women	Men	Women
Gp1	5	8	115.8	79.4	38.0	31.4	1.1	1.1
Gp2	4	9	83.3	92.7	28.4	33.9	1.3	1.1
Gp3	7	7	85	79.2	26.2	27.8	1.0	1.1
Gp4	6	7	93.8	75.7	30.9	31.5	1.1	1.1

4.2.4 Study Treatment Randomisation

After the initial randomisation, blinding was not possible to those applying the investigational product, because the characteristics of the study product DG, could be easily distinguished from the polyglactin mesh alone. The mesh alone may also interact with the wound to such an extent that it could make interpretation of the results difficult. Consequently, patients with DG plus multi-layer compression bandage therapy versus a control group of patients who received multi-layer compression bandage therapy only were compared.

Compliance was assured both in the application of DG and with the multi-layer compression therapy, as these occurred in the controlled setting of a clinic in each centre.

53 patients entered the randomised, controlled phase of the study. The distribution of these patients into the four groups, centre by centre is seen in Table 4-10.

Table 4-10 Distribution of patients in the four treatment groups

	Randomisation Group				
Centre	**1**	**2**	**3**	**4**	**Total**
Cardiff	6	7	7	6	**26**
Cheltenham	2	2	2	2	**8**
Stockton-on-Tees	1	1	1	1	**4**
Toronto/ Mississauga	2	2	2	2	**8**
Montreal	2	1	2	2	**7**
Winnipeg	0	0	0	0	**0**
Total	**13**	**13**	**14**	**13**	**53**

The mean size of the target ulcer was similar between the four groups, but the duration of the ulcer varied between the four groups. This is shown in Table 4-11

Table 4-11 Duration of ulceration and size of ulcer, which is similar in the four treatment groups

	Group1	Group2	Group3	Group4
Median ulcer size at Week 0 (cm^2) [Range]	8.6 [6.1–22]	5.6 [3.6–20.2]	6.8 [3.7–25]	9.2 [3.7-25]
Median duration of target ulcer (wks) [Range]	34.7 [13–260]	52.0 [9–260]	43.3 [12–239]	73.7 [9–260]

After randomisation, 6 patients withdrew from the study, of these 1 patient developed wound infection, 1 patient developed severe excoriation from the four-layer bandage, 3 patients stopped using the compression bandages, and 1 patient did not receive DG.

Thus 47 patients completed the study as per protocol. Table 4-12.

Table 4-12 Reason for withdrawal after randomisation

Centre	Group	Reason for Withdrawal
Cardiff	2	Wound infection
Cardiff	3	Excoriation to bandages
Cardiff	3	Patient withdrew consent
Cheltenham	3	Non-compliance with compression
Cheltenham	3	Non-compliance with compression
Montreal	3	Did not receive DG

4.2.5 Biopsy procedure

Three patients refused the second biopsy but were followed up until completion of the study to clinically assess the progress of the target ulcer towards healing, and four week 6 biopsies were of inadequate depth to perform valuable histological evaluations.

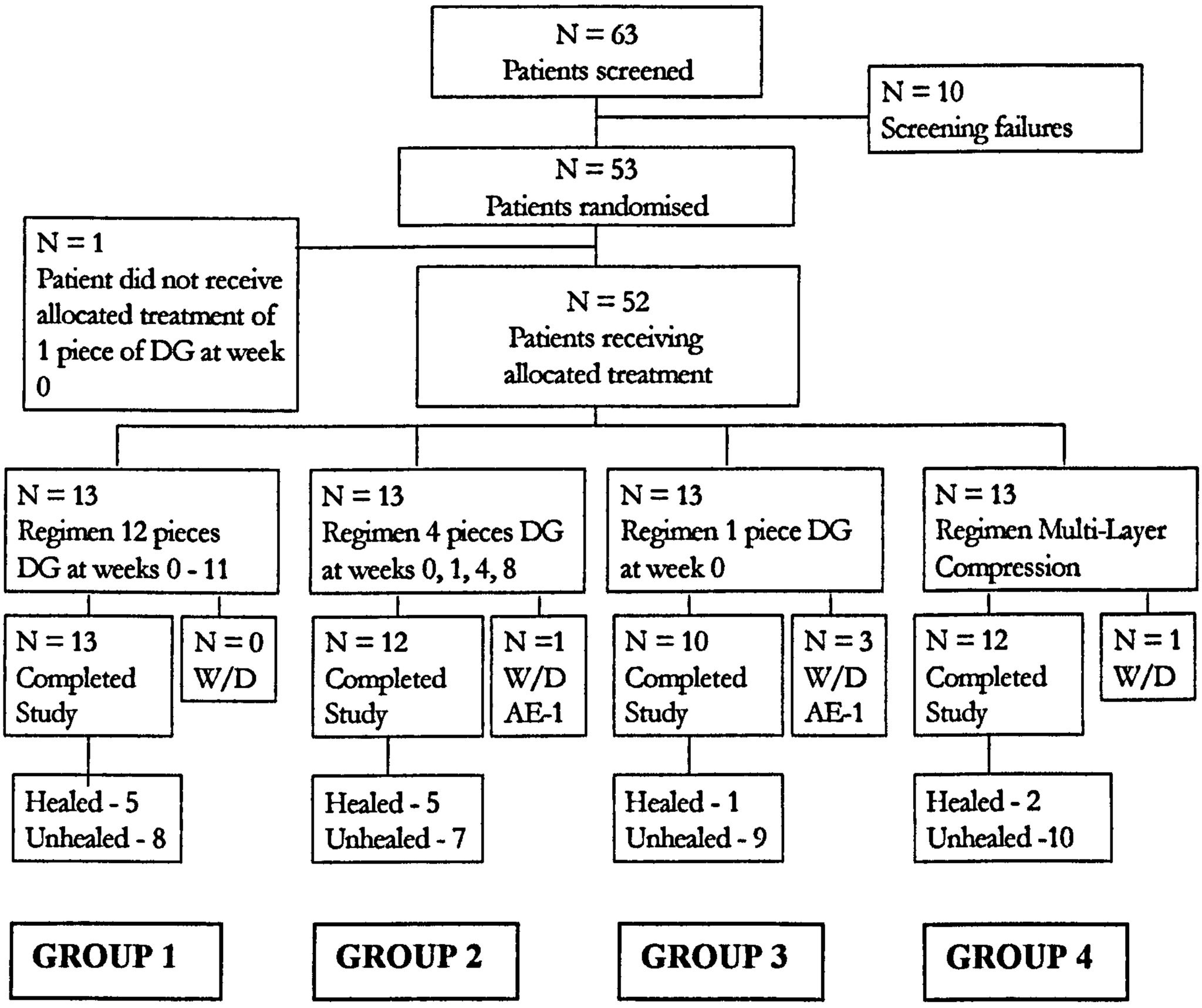
4.2.6 Assessment of Healing

4.2.6.1 Clinical Assessment

Photographs of the wound and clinical assessment of the ulcer and patient were useful in identifying early adverse events during the study period.

Figure 4-8 shows the disposition of all patients and the progress through the phases of the trial.

Figure 4-8 Algorithim of Sample Size and Disposition of all Patients in the Main Study



4.2.6.2 Histological Assesment

4.2.6.2.1 *Wax sections from patient biopsy*

The ethanol preserved part of the biopsies were mounted in wax, sectioned, and stained in the laboratories at the Smith and Nephew, Group Research Centre, York, UK. The stained slides were analysed by a researcher at Smith and Nephew, York and by an experienced Histopathologist with the investigator. Out of the 47 patients who completed the study, 35 paired biopsies were analysed at York and 33 at Cardiff. The main cause of non-evaluable biopsies was insufficient biopsy depth in some as they only contained wound eschar. The shallow biopsies were subsequently traced to specimens obtained from peripheral centres and not by the investigator at the Cardiff centre. The second cause of non-evaluable biopsies was tissue destruction during processing of the sections. This was done by Smith and Nephew, UK (York), using an automated method which resulted in heat damage to one set of slides.

4.2.6.2.2 *Frozen sections from patient biopsy*

Out of the 47 patients who completed the study, 40 pairs of biopsies were analysed. A second sample was not obtained from 3 patients, and in the remaining four sets the biopsies were too shallow to allow any meaningful analysis.

4.2.7 Outcome Analysis

4.2.7.1 Clinical

Of the 47 patients who completed the study, 13 patients (27.7%) healed during the course of the study, and 34 patients (72.3%) had an ulcer remaining at the end of the study period. (Table 4-13)

Table 4-13 Randomised patients and their disposition over the course of the study

Centre No	Randomisation Group								Total		Overall
	1		2		3		4				
	H	U	H	U	H	U	H	U	H	U	Totals
1	1	5	2	4	0	5	1	5	4	19	**23**
2	1	1	40	2	0	1	1	0	2	4	**6**
3	0	1	1	0	1	0	0	1	2	2	**4**
4	1	1	2	0	0	2	0	2	3	5	**8**
5	2	0	0	1	0	1	0	2	2	4	**6**
Totals	**5**	**8**	**5**	**7**	**1**	**9**	**2**	**10**	**13**	**34**	**47**

(H = healed: U = unhealed)

4.2.7.2 Efficacy

4.2.7.2.1 *Number of patients healed in the 12 week study period*

The difference between the treatment groups (1, 2 and 3) was not significant (Fisher's exact test). When groups 1 and 3, and, groups 2 and 3 respectively were compared, they approached significance with a $p = 0.06$. The small number of patients in the study groups may have a contributory factor in the statistical outcome.

However, when compared to Control (group 4), a higher proportion of patients healed in groups 1 and 2, than group3. (Table 4-14)

Table 4-14 Distribution of healing in the treatment groups

	STUDY POPULATION			
	Group 1 (n = 13)	**Group 2 (n = 13)**	**Group 3 (n = 14)**	**Group 4 (n = 13)**
Median Ulcer Size at Week 0 (cm^2)	8.6	5.6	6.8	9.2
Median Duration of Ulceration (weeks)	34.7	52.0	43.3	73.7
No. of Ulcers Healed at Week 12	5/13 (38%)	5/13 (38%)	1/14 (7%)	2/13 (15%)

4.2.7.2.2 *Time to wound closure*

Ulcer Area

At the end of the study period i.e. at 12 weeks, the median reduction in ulcer area was 81.4% for group 1, 88.6% for group 2, 59.4% for group 3, and 78.1% for group 4. (Table. 4-15)

Table 4-15 Percentage Reduction in Wound Area at week 12 of Study

% Reduction in Area	Group 1	Group 2	Group 3	Group 4
Mean	68.1	58.2	41.1	32.1
Median	81.4	88.6	59.4	78.1
Std	39.2	63.7	60.5	39.2
Minimum	-11.3	-119.1	-117.7	-21.7
Maximum	100	100	100	100
Number	13	13	14	13

The healing rates for each treatment group when compared to the baseline ulcer area and duration demonstrated that ulcers up to $10cm^2$ that had been present for up to 1 year prior to the study was 60% (3 out of 5) for group 1, 57% (4 out of 7) for group 2, 0% (0 out of 4) for group 3, and 33% (1 out of 3) for group 4. (Table. 4-16)

Table 4-16 Number of patients healed by week 12 with small ulcers present for < 1 year

Area cm^2	**Gp 1**	**Gp 2**	**Gp 3**	**Gp 4**
3 – 5.0	H = 0 U= 0	H = 2 U = 1	H = 0 U = 3	H = 0 U = 2
5.01 – 7.5	H = 1 U = 2	H = 1 U = 2	H = 0 U = 1	H = 1 U = 0
7.51 – 10.0	H = 1 U = 0	H = 1 U = 0	H = 0 U = 1	H = 0 U = 0
Total (healed)	3/5	4/7	0/4	1/3
Percentage	60	57	0	33

(H = Healed, U = Unhealed)

Even when all ulcer sizes were grouped together (maximum of $25cm^2$) present for up to a year, the healing rates are shown to be 62.5% (5 out of 8) for group 1, 44% (4 out of 9) for group 2, 12.5% (1 out of 8) for group 3, and 20% (1 out of 5) for group 4. (Table.4-17)

Table 4-17 Number of patients healed by week 12 for all ulcer areas present for < 1 year

Area cm^2	Gp 1	Gp 2	Gp 3	Gp 4
3 – 5	H = 1 U = 0	H = 2 U = 1	H = 0 U = 3	H = 0 U = 2
5.01 – 7.5	H = 1 U = 2	H = 1 U = 2	H = 0 U = 1	H = 1 U = 0
7.51 – 10.0	H = 1 U = 0	H = 1 U = 0	H = 0 U = 1	H = 0 U = 0
10.1 – 15.0	H = 1 U = 1	H = 0 U = 0	H = 0 U = 1	H = 0 U = 1
15.01 – 26.0	H = 1 U = 0	H = 0 U = 2	H = 1 U = 2	H = 0 U = 2
Total	5 /8	4/9	1/ 8	1/5
Percentage	62.5	44	12.5	20

(H = Healed, U = Unhealed)

This demonstrates that even in patients with large ulcers, wound closure is achievable if it has been present for less than 1 year.

If the ulcer has been present for more than a year, the healing potential of the wound seems to be greatly reduced, however some healing is seen in smaller wounds i.e those less than $10cm^2$. (Table 4-18)

Table 4-18 Number of patients healed by week 12 for all ulcer areas present for > 1 year

Area cm^2	Gp 1	Gp 2	Gp 3	Gp 4
3 – 5	H = 0 U = 0	H = 1 U = 1	H = 0 U = 2	H = 0 U = 1
5.01 – 7.5	H = 0 U = 2	H = 0 U = 1	H = 0 U = 2	H = 0 U = 2
7.51 – 10.0	H = 0 U = 0	H = 0 U = 1	H = 0 U = 0	H = 1 U = 0
10.1 – 15.0	H = 0 U = 1	H = 0 U = 0	H = 0 U = 0	H = 0 U = 2
15.01 – 26.0	H = 0 U = 2	H = 0 U = 0	H = 0 U = 1	H = 0 U = 1
Total	0/5	1/4	0/5	1/6
Percentage	0	25	0	16.7

(H = Healed, U = Unhealed)

4.2.7.2.3 *Adverse Patient Events*

The adverse events were reported according to medical device guidelines in Canada, and according to pharmaceutical guidelines in the UK. 80 episodes of adverse events were recorded for the 63 patients who were initially screened for the study. Of the 53 patients who were eventually randomised into the study groups a total of 65 adverse events were reported in 33 patients, and 20 patients being unaffected. The adverse events ranged from allergic reactions to DG or the secondary dressings, to more serious conditions such as Pemphigoid and Cerebro-vascular accident (CVA).

The most commonly reported adverse event was infection, accounting for 49% of the total events; this was seen in 43% (23 out of the 53 randomised patients). (Table 4-19) However when the adverse events were broken down into the four treatment groups , it was observed that there were no differences in the incidence of infection in the four treatment groups. (Table 4-20). No deaths were reported during the duration of the study.

Table 4-19 Distribution of adverse effects encountered during the course of the study.

Adverse Event	**No. Reported (%) (n = 65)**	**No. Patients (%) (n = 53)**
Allergy	1 (2%)	1 (2%)
Contact Dermatitis	6 (9%)	6 (11%)
Cellulitis	5 (8%)	5 (9%)
Cerebro-vascular accident	1 (2%)	1 (2%)
Eczema	13 (20%)	11 (21%)
Erythema	2 (3%)	2 (4%)
Infection	32 (49%)	23 (43%)
Fungal infection	1 (2%)	1 (2%)
Injury	2 (3%)	2 (4%)
Hypoglycaemic reaction	1 (2%)	1 (2%)
Pain	2 (3%)	2 (4%)
Pemphigoid	1 (2%)	1 (2%)
Pruritis	1 (2%)	1 (2%)
Skin injury	4 (6%)	4 (8%)
Skin maceration	2 (3%)	2 (4%)
Surgical complication	4 (6%)	4 (8%)
Syncope	1 (2%)	1 (2%)
Wound deterioration	1 (2%)	1 (2%)

Table 4-20 Reported adverse events in the four treatment groups

Adverse event	Group 1 (%) **(n = 13)**	Group 2 (%) **(n = 13)**	Group 3 (%) **(n = 13)**	Group 4(%) **(n = 13)**
Allergy	1 (8%)	0	0	0
Cellulitis	1(8%)	2 (15%)	0	1 (8%)
Dermatitis	2(15%)	1 (8%)	1 (8%)	4 (31%)
Erythema	1 (8%)	0	0	1 (8%)
Infection	4 (31%)	3 (23%)	4 (31%)	6 (46%)
Pemphigoid	1 (8%)	0	0	0
Pruitis	1 (8%)	0	0	0
Skin excoriation	2(15%)	1 (8%)	3 (23%)	1 (8%)
Skin injury	1 (8%)	1 (8%)	2 (15%)	1 (8%)
Skin maceration	1 (8%)	1 (8%)	0	0
Wound deterioration	0	1 (8%)	0	0
Surgery complication	0	0	2 (15%)	0
Syncope	0	0	1 (8%)	0
Hypoglycaemia	0	0	0	1 (8%)
Pain	0	2 (15%)	0	0
TOTAL	18	15	15	17

Histological Analysis

4.2.8 Wax sections

All microscopic sections were full or partial thickness skin biopsies, most with a fully ulcerated surface, and a few possessing epithelium at the edge or in the centre. These epithelised areas appeared to be neoepithelium rather than pre-existing epidermis. Most biopsies had a surface exudate of fibrin and other eosinophillic material with a neutrophil infiltrate. Beneath this was a zone of granulation tissue supporting the blood vessels.

The % reduction in the wound area at weeks 6 and 12, and the total number of wounds healed were compared against the three histological parameters at weeks 0 and 6.

4.2.8.1 Extracellular matrix (ECM) and collagen distribution

There were no statistically significant (Fisher's exact test) association between the ECM levels and healing, but there were some weak associations. A low matrix score (i.e one which had the least amount of macromolecules in the ECM) at week 6 was related to increased numbers of healed wounds at week 12 ($p = 0.06$) and in the percentage reduction of the wound area at week 12 ($p = 0.08$). A decrease in the matrix score between weeks 0 and 6 was related to healing, and an increase in the matrix score in the same two time periods related to the non-healing nature of the ulcers ($p = 0.09$).

A patchy initial distribution of the ECM at week 0 (i.e. a low matrix score) was associated with the percentage reduction in the ulcer area at week 12 ($p = 0.08$) and patchy distribution of the ECM at weeks 0 and 6 was linked with increased numbers of healed wounds at week 12 ($p = 0.096$, and $p = 0.031$, respectively). Comparing the treatments groups with the control group, only patients in group 2 (i.e. those who received 4 pieces of Dermagraft), demonstrated a reduction in the ECM score between weeks 0 and 6 when compared to the control group ($p = 0.048$).

There was no significant relationship between the depth of granulation tissue at weeks 0, 6, and changes in the granulation tissue depth between weeks 0 and 6 to the healing of the ulcer. The only interesting finding was the mean depth of granulation tissue in group 1 (i.e. those who received 12 pieces of Dermagraft) at week 0 was significantly less than the mean depth in the control group ($p = 0.01$), however this discrepancy had resolved in the week 6 sections.

With regards to collagen pattern seen within the wound bed 1 mm from the surface, no link was seen between the original or changes in pattern of collagen between the treatment groups and control group at weeks 0, week 6, or its change from week 0 to week 6, with healing or percentage reduction of the wound.

Only 23 pairs of biopsies were suitable for the assessment collagen depth at the base of the biopsies. 24 pairs of biopsies were too shallow and did not incorporate the base of the wound. All 23 pairs showed the fibres running parallel to the surface of the wound rather than the basket weave pattern that is normally seen in uninjured skin.

High levels of collagen deposit defined as a 'Dense Scar' in the week 0 sections correlated with a significant reduction in the area of the ulcer at the week 6 time point ($p = 0.03$) and in the complete closure of the wound at week 12 ($p = 0.02$), but there were no significant differences between the 4 groups of the study.

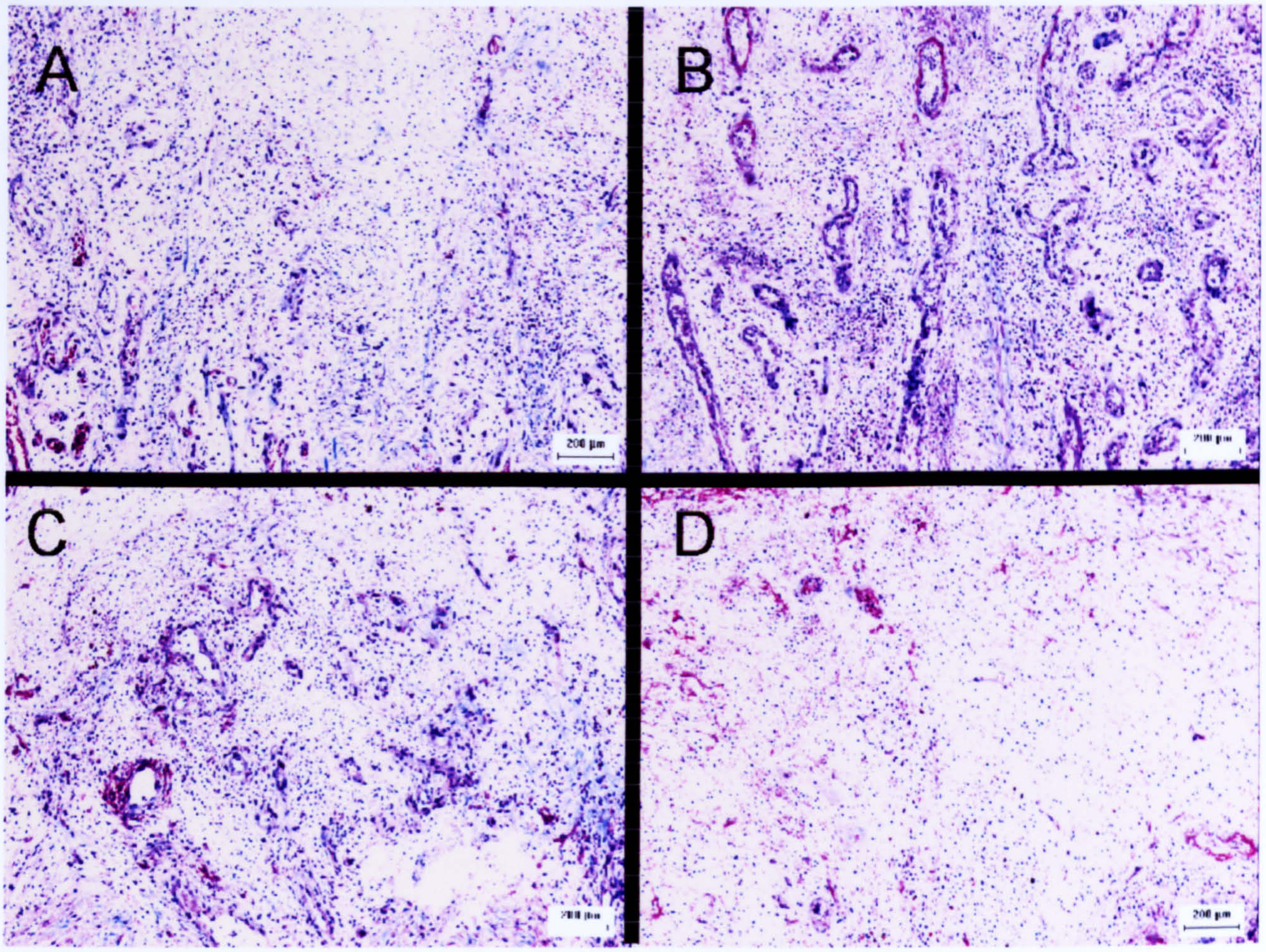
4.2.8.2 Blood vessels

The number of vascular units/mm^2 was counted in the whole section. These units were usually arranged in clusters of proliferating capillaries like an angioma, fed from a vessel in the deep dermis. It was noted that some of these units were hyperplastic with large numerous capillaries whilst others hypoplastic.

Large numbers of blood vessels in the week 0 or week 6 correlated well in the number of healed wounds at week 12 ($p = 0.06$, $p = 0.04$). Large numbers of blood vessels in the week 6 biopsies correlated well in the percentage reduction of the wound area at week 12 ($p = 0.05$). (Figure 4-9)

Figure 4-9 Distribution of Blood Vessels Within the Wound Bed at Weeks 0 and 6

Patient 1 at Week 0 (A) and Week 6 (B) - increased number of blood vessels is observed which correlated to healing

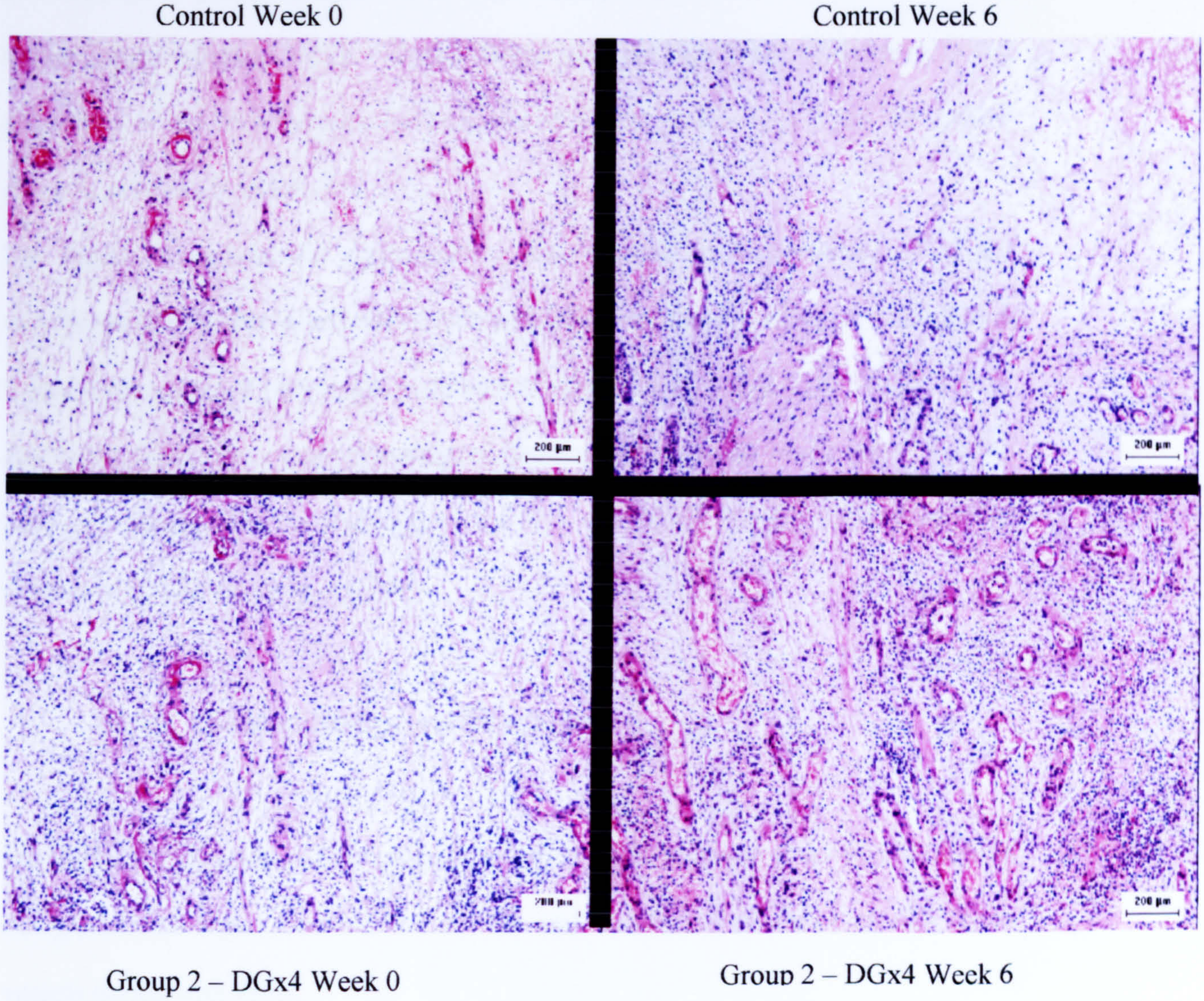


Patient 2 at Week 0 (C) and Week 6 (D) –decreased number of blood vessels is observed which correlated to no healing.

When comparing the treatment groups, group 2 (i.e 4 pieces of DG) demonstrated a significantly greater numbers of blood vessels at week 6 than the control group ($p<0.05$). This level of difference was not seen with patients given 1or 12 pieces of DG. Figure 4-8 illustrates a paired biopsy from a patient in the control group and a patient from group 2 (DGx4). The top paired photomicrograph (the control group) demonstrated no observable increase in the number of blood vessels at week 6 when compared to week 0, and the bottom paired photomicrograph (DGx4). demonstrated an increase in the number of blood vessels at week 6 when compared to week 0 (Figure 4-10)

Figure 4-10 Increase in the Number of Blood Vessels Stimulated by Dermagraft (4 Pieces)

Control patient at Week 0 and Week 6 – negligible change in the number of blood vessels is observed



DG x 4 patient at Week 0 (A) and Week 6 (B) – increase in the number of blood vessels is observed

Vessel orientation appears to play a role in the healing of the ulcers. The vessels were classified as either perpendicular or randomly distributed throughout the tissue. A random distribution of vessels at week 6 seems to be related with an increased reduction in the ulcer size at week 12 ($p = 0.06$), and a change in the vessel orientation from random at week 0 to a perpendicular configuration at week 6 seems to be related to poor healing at week 6 ($p = 0.09$) and a decrease in the number of healed wound at week 12 ($p = 0.03$). There were no significant differences in the orientation of the blood vessels between the treatment groups 1, 2, 3 and the control group 4, at weeks 0 and 6.

The degree of perivascular fibrin cuffing was assessed as a percentage of all the vessels present. It was noted that a low percentage of fibrin cuffing at week 0 was linked with increased percentage reduction of ulcer size at weeks 6 ($p < 0.05$) and 12 ($p < 0.05$). Similarly, a low percentage of fibrin cuffing at week 6 was linked with an increased reduction in the ulcer size at weeks 6, ($p = 0.05$), 12 ($p = 0.05$), and in the number of healed ulcers ($p = 0.05$).

4.2.8.3 Cellular Pattern and Distribution

Neutrophil polymorphs and lymphocytes were the most common types of cells seen in the granulation tissue between the vessels and sometimes clustered around blood vessels cuffed with fibrin.

Out of the 47 patients who entered the study, week 0 and week 6 sections stained with the neutrophil antibody were available from only 33 patients.

At week 0 the mean number of neutrophils in the four groups is shown in Table 4-21.

Table 4-21 The mean count of neutrophils at T = 0 within the four groups

Groups	Week 0 Mean (SD)
Gp1 (DGx12)(n = 8)	99.8 (48.7)
Gp2 (DGx4)(n = 9)	139 (62.5)
Gp3 (DGx1)(n = 6)	125.5 (49.0)
Gp4 (Control)(n = 10)	140.1 (40.5)

Biopsies obtained from patients randomised to the Control group (Group 4) demonstrated a higher proportion of neutrophils than Group 1 ($p = 0.01$), Group 2 ($p = 0.03$), or Group 3 ($p = 0.02$) at week 0. However this difference was not repeated in the week 6 sections.

In all groups, the mean count of neutrophils at week 6 tended to be less than the mean count at week 0, but this reduction in the counts were not statistically significant, except in the control group ($p<0.05$).(Table 4-22 and Figures 4-11, 4-12)

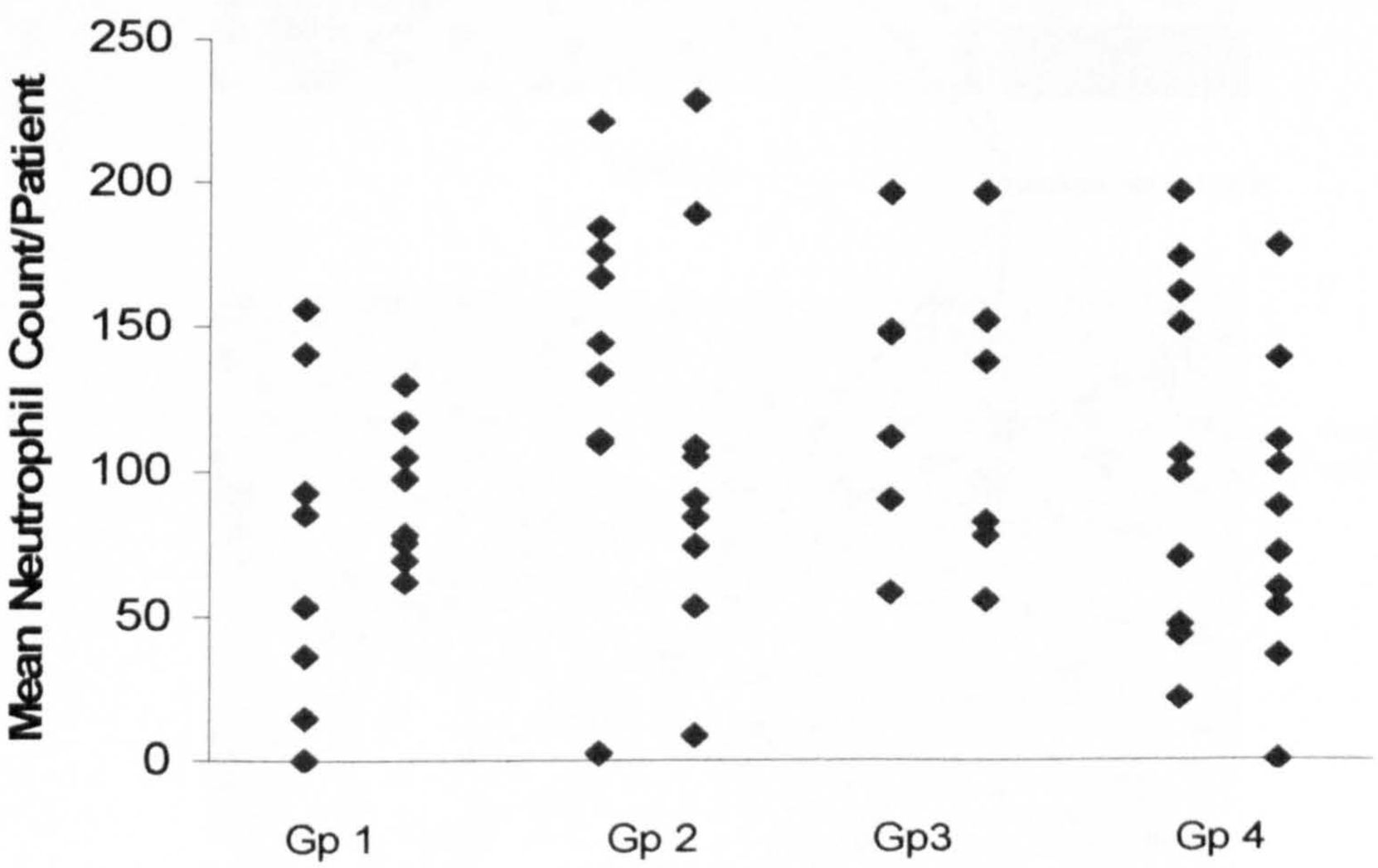
The large SD in all groups may suggest patient variability within the groups.

Table 4-22 The mean count of Neutrophils within chronic wounds at T = 0 and

T = 6 weeks

Groups	Week 0 Mean (SD)	Week 6 Mean (SD)	Mann – Whitney U P values
Gp1 (DGx12)	99.8 (48.7)	91.4 (24.2)	0.5
Gp2 (DGx4)	139 (62.5)	104.2 (67.0)	0.16
Gp3 (DGx1)	125.5 (49.0)	117.3 (54.0)	0.81
Gp4 (Control)	140.1 (40.5)	83.8 (51.9)	0.02

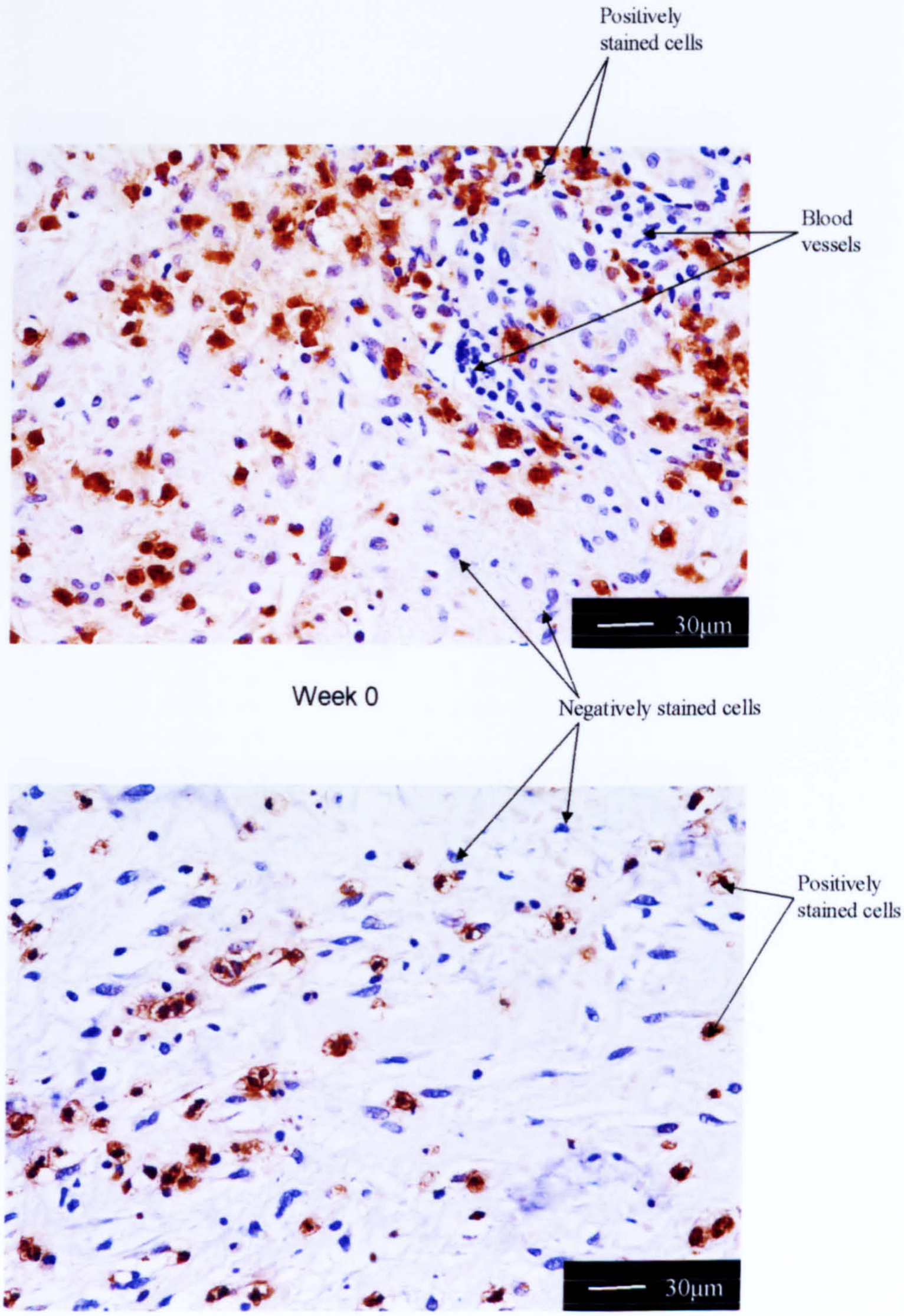
Figure 4-11 Mean Neutrophil Counts in Chronic Wounds at T=0 and T=6 weeks for the Four Groups.



In all groups, the mean count of neutrophils at week 6 tended to be less than the mean count at week 0, but this reduction in the counts were not statistically significant,

Figure 4-12 Photomicrographs of Neutrophil Distribution at Weeks 0 and 6 in the Four Treatment Groups

Group 1 (DGx12)

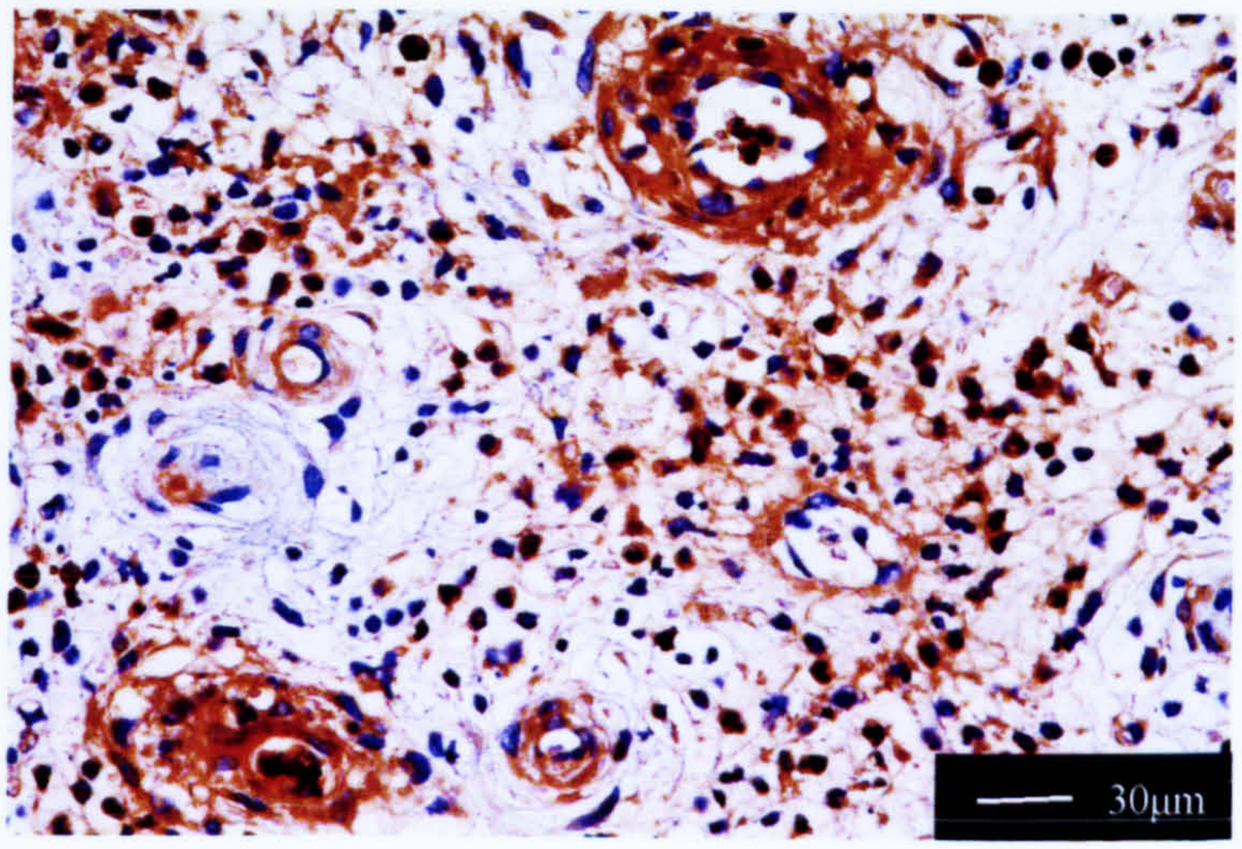


Week 0

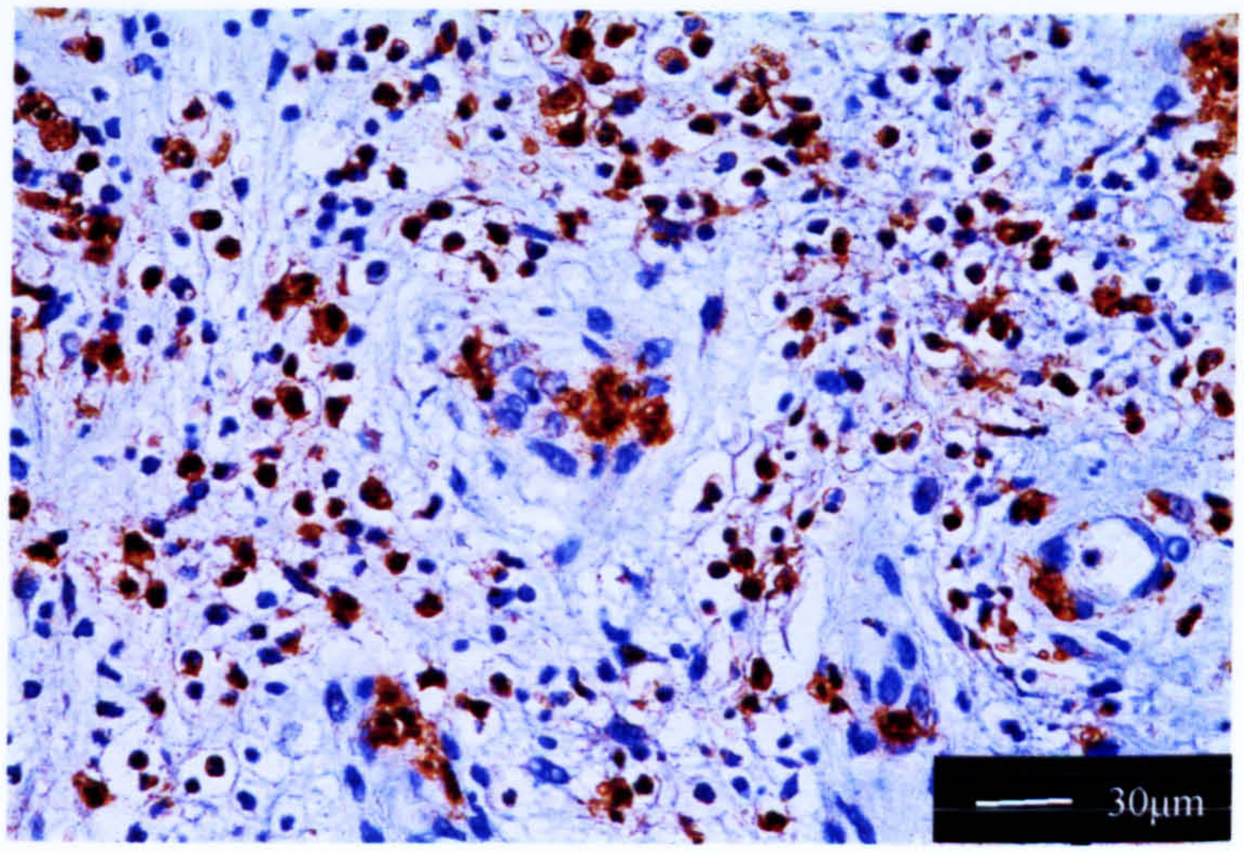
Week 6

There is a decrease in the mean count of neutrophils at week 6 when compared to week 0.

Group 2 (DGx4)



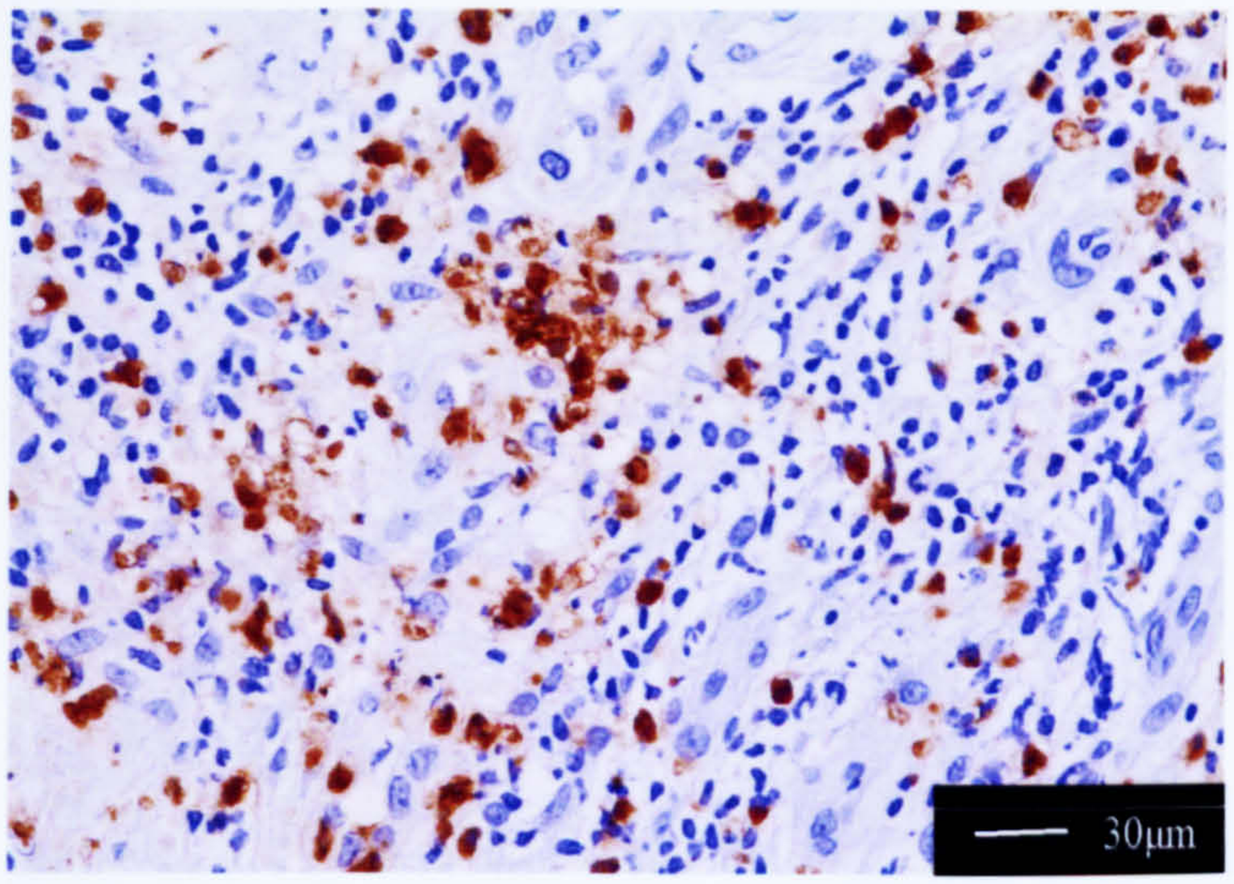
Week 0



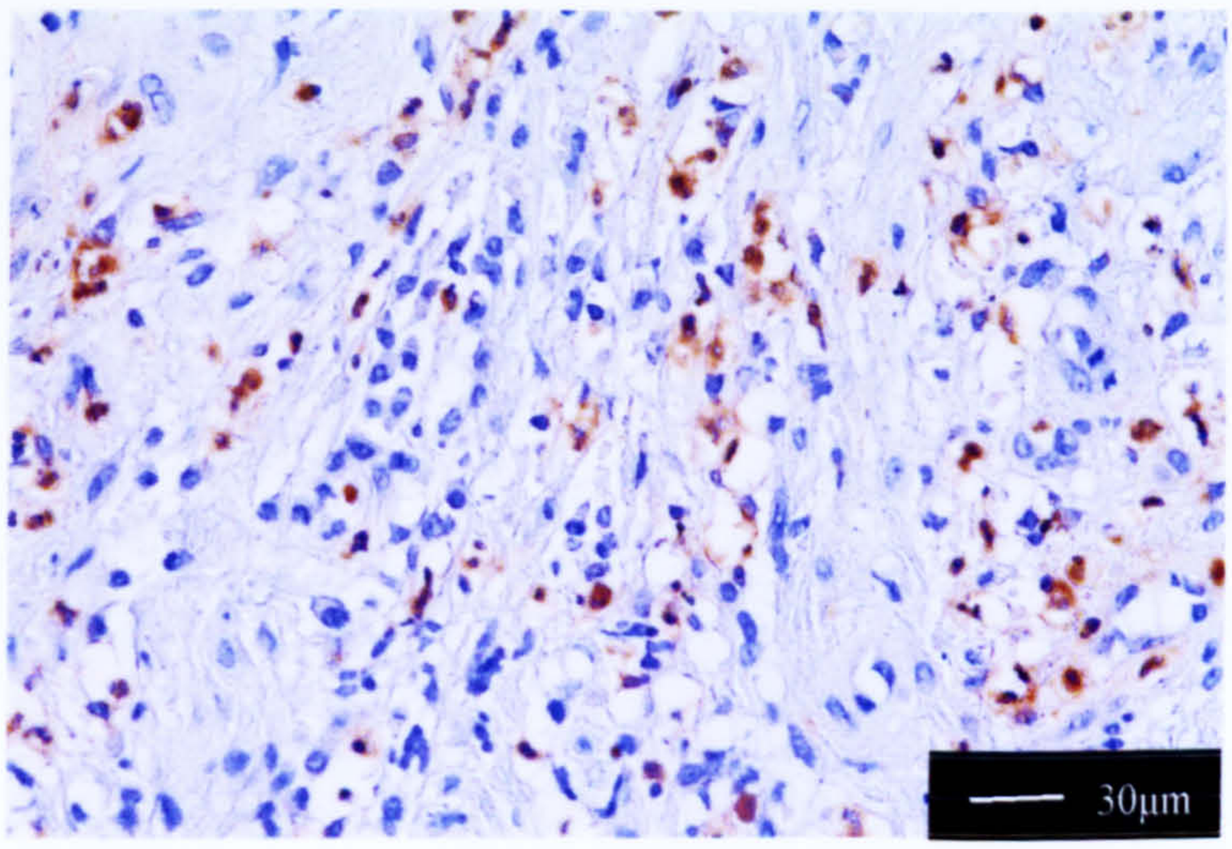
Week 6

There is a decrease in the mean count of neutrophils at week 6 when compared to week 0.

Group 3 (DGx1)



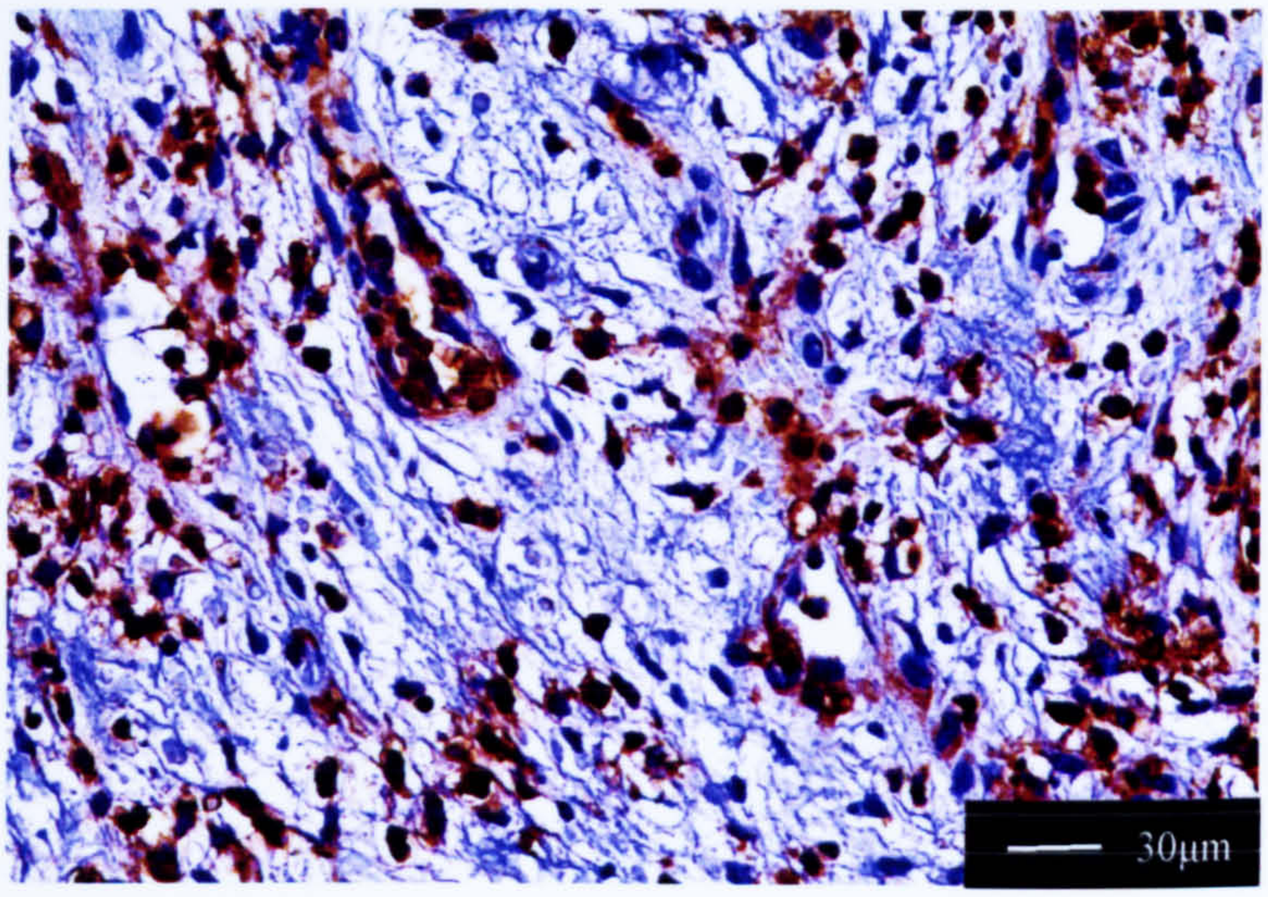
Week 0



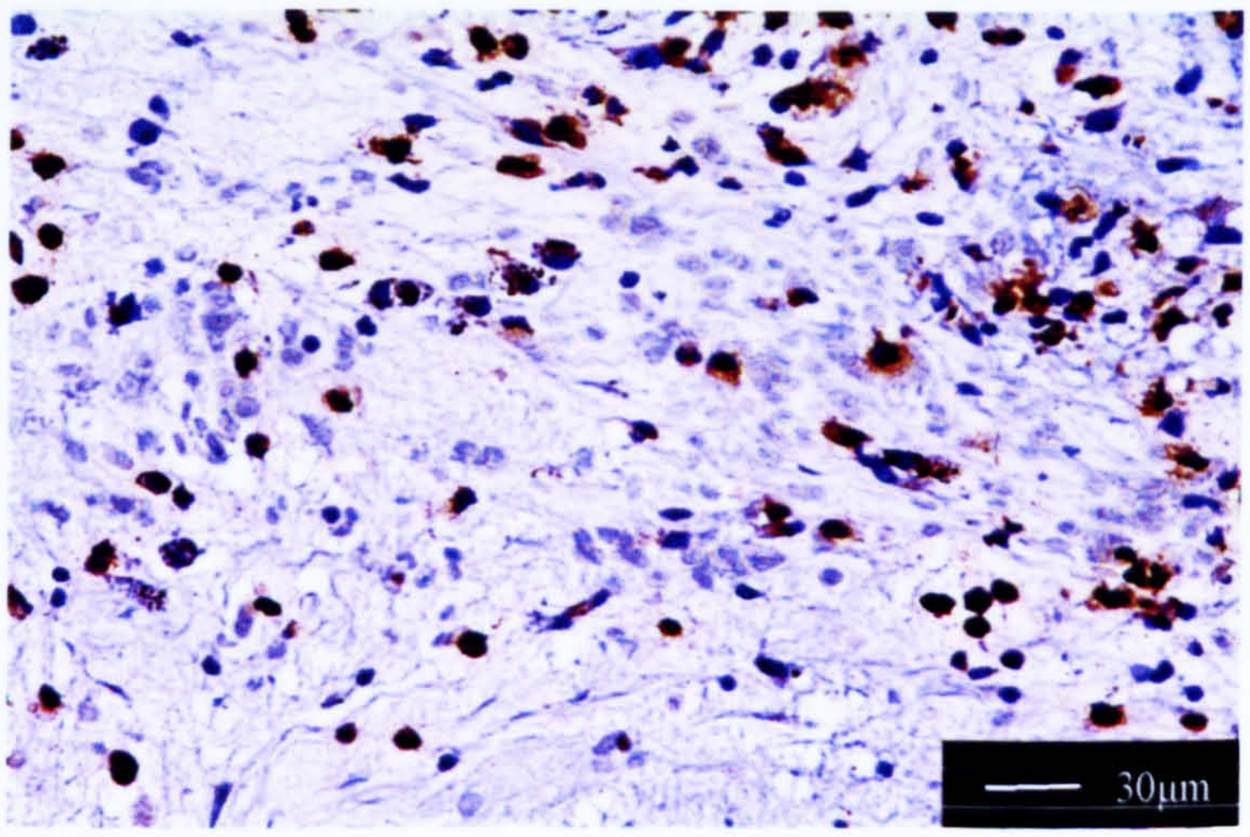
Week 6

There is a decrease in the mean count of neutrophils at week 6 when compared to week 0.

Group 4 (Control)



Week 0



Week 6

There is a decrease in the mean count of neutrophils at week 6 when compared to week 0, which is statisically significant, but the SD in this group is large suggesting a large patient variablilty

4.2.9 Frozen sections

4.2.9.1 Inflammatory Cells

4.2.9.1.1 *Lymphocytes*

Cells of the lymphocytic morphology, B- and T- lymphocytes are small round cells with little cytoplasm and a round nucleus, were preferentially found in the peri-vascular areas.

<u>B-Lymphocytes</u>

The CD19 antigen is the most broadly expressed surface marker for B-lymphocytes (Dorken B et al., 1992a).

The mean count of B-lymphocytes between the four groups at Week 0 seems to be of similar magnitude. This suggests that there is a similar distribution of B-lymphocytes at Week 0 within the four treatment groups. (Table 4-23)

Table 4-23 The mean count of B lymphocytes at Week 0 within the four groups

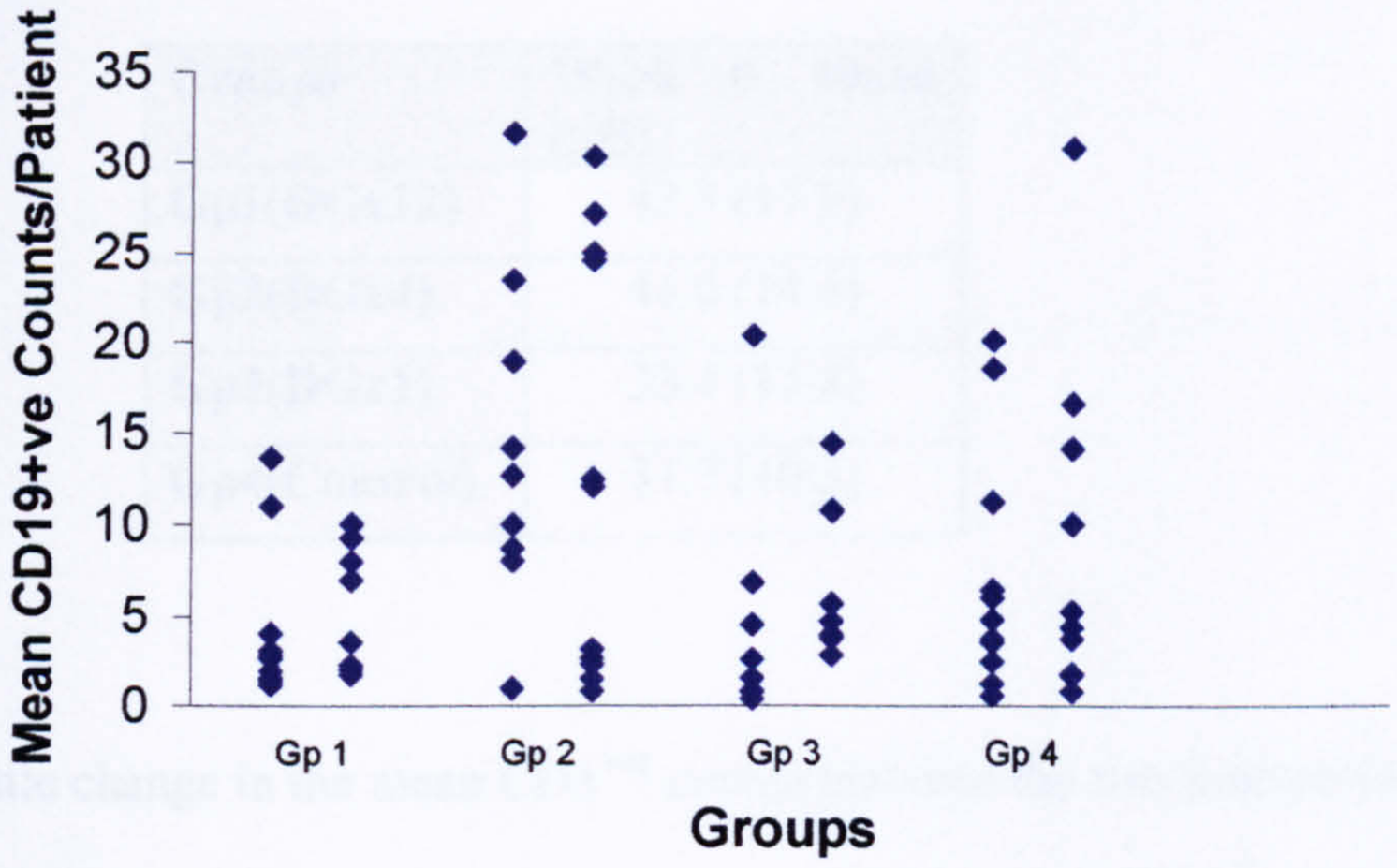
Groups	Week 0 Mean (SD)
Gp1(DGx12)	4.4 (4.3)
Gp2(DGx4)	12.8 (7.7)
Gp3(DGx1)	4.7 (3.6)
Gp4(Control)	7.1 (5.0)

There seems to be very little change in the mean count of B-Lymphocytes at Week 6 when compared to the mean count at Week 0 across all four groups. (Table. 4-24, Figure 4-13)

Table 4-24 The mean count of $CD19^{+ve}$ B lymphocytes within chronic wounds at Week 0 and Week 6

Groups	Week 0 Mean (SD)	Week 6 Mean (SD)	Mann Whitney-U P values
Gp1(DGx12)	4.4 (4.3)	5.6 (4.4)	0.35
Gp2(DGx4)	12.8 (7.7)	15.2 (6.2)	0.85
Gp3(DGx1)	4.7 (3.6)	7.1 (6.5)	0.10
Gp4(Control)	7.1 (5.0)	8.2 (4.90	0.95

Figure 4-13 Mean CD19^{+ve} B-Lymphocyte Counts in Chronic Wounds at Week 0 and Week 6 for the Four Groups.



There seems to be very little change in the mean count of B-Lymphocytes at Week 6 when compared to the mean count at Week 0 across all four groups

<u>T-Lymphocytes</u>

Like B-lymphocytes, T-lymphocytes were identified in their greatest numbers in the peri-vascular areas. T-Lymphocytes were identified by the pan lymphocytes marker - CD3, T helper/inducer lymphocytes - CD4, and T suppressor/cytotoxic lymphocytes - CD8.

There is no difference in the mean count of T-lymphocytes as identified by staining positive with anti-CD3 monoclonal antibody between the four groups at the start of the study (Week 0). (Table. 4-25)

Table 4-25 The mean count of $CD3^{+ve}$ T-lymphocytes at Week 0 within the four groups

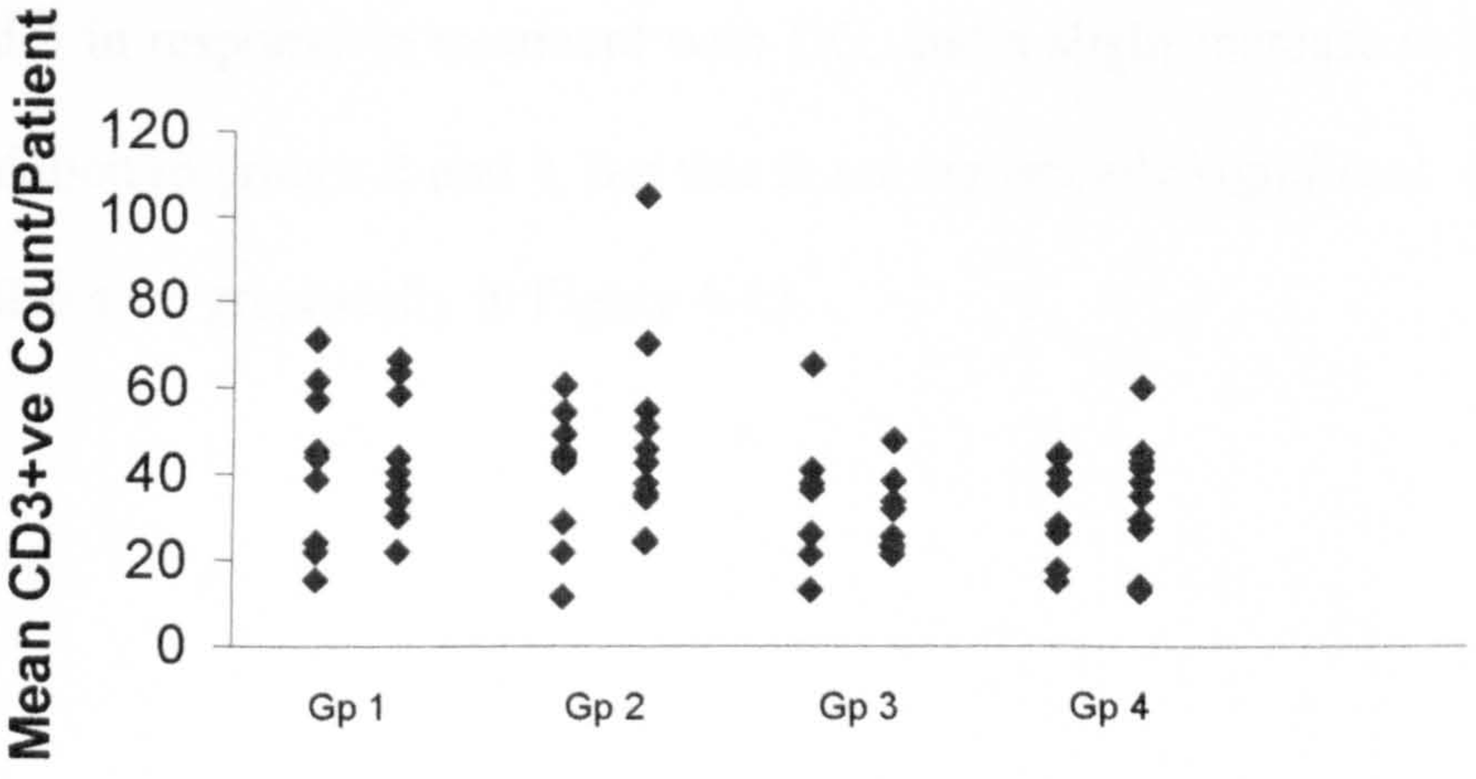
Groups	Week 0 Mean (SD)
Gp1(DGx12)	42.5 (17.9)
Gp2(DGx4)	41.0 (14.4)
Gp3(DGx1)	33.4 (15.8)
Gp4(Control)	31.7 (10.3)

There is little change in the mean $CD3^{+ve}$ counts between the two time periods (Week 0 and Week 6) in groups 1 and 3, but there is a small non-significant increase in the mean $CD3^{+ve}$ counts between weeks 0 and 6 in groups 2 and 4 in response to treatment. (Table 4-26, Figure 4-14)

Table 4-26 The mean count of $CD3^{+ve}$ T-Lymphocytes within chronic wounds at Week 0 and Week 6

Groups	Week 0 Mean (SD)	Week 6 Mean (SD)	Mann – Whitney U P values
Gp1(DGx12)	42.5 (17.9)	43.1 (14.8)	0.68
Gp2(DGx4)	41.0 (14.4)	47.8 (23.1)	0.60
Gp3(DGx1)	33.4 (15.8)	30.0 (9.7)	0.72
Gp4(Control)	31.7 (10.3)	39.1 (9.9)	0.60

Figure 4-14 Mean $CD3^{+ve}$ T-Lymphocyte Count in Chronic Wounds at Week 0 and Week 6



There is a small non-significant increase in the mean $CD3^{+ve}$ counts between weeks 0 and 6 in groups 2 and 4 in response to treatment.

Similarly, the mean count of helper/inducer T-lymphocytes (a sub-population of T-lymphocytes), as identified by staining positive with anti-CD4 monoclonal antibody, exhibits no gross variation between the four groups at the start of the study at Week 0. (Table 4-27)

Table 4-27 The mean count of $CD4^{+ve}$ T-lymphocytes at Week 0 within the four groups

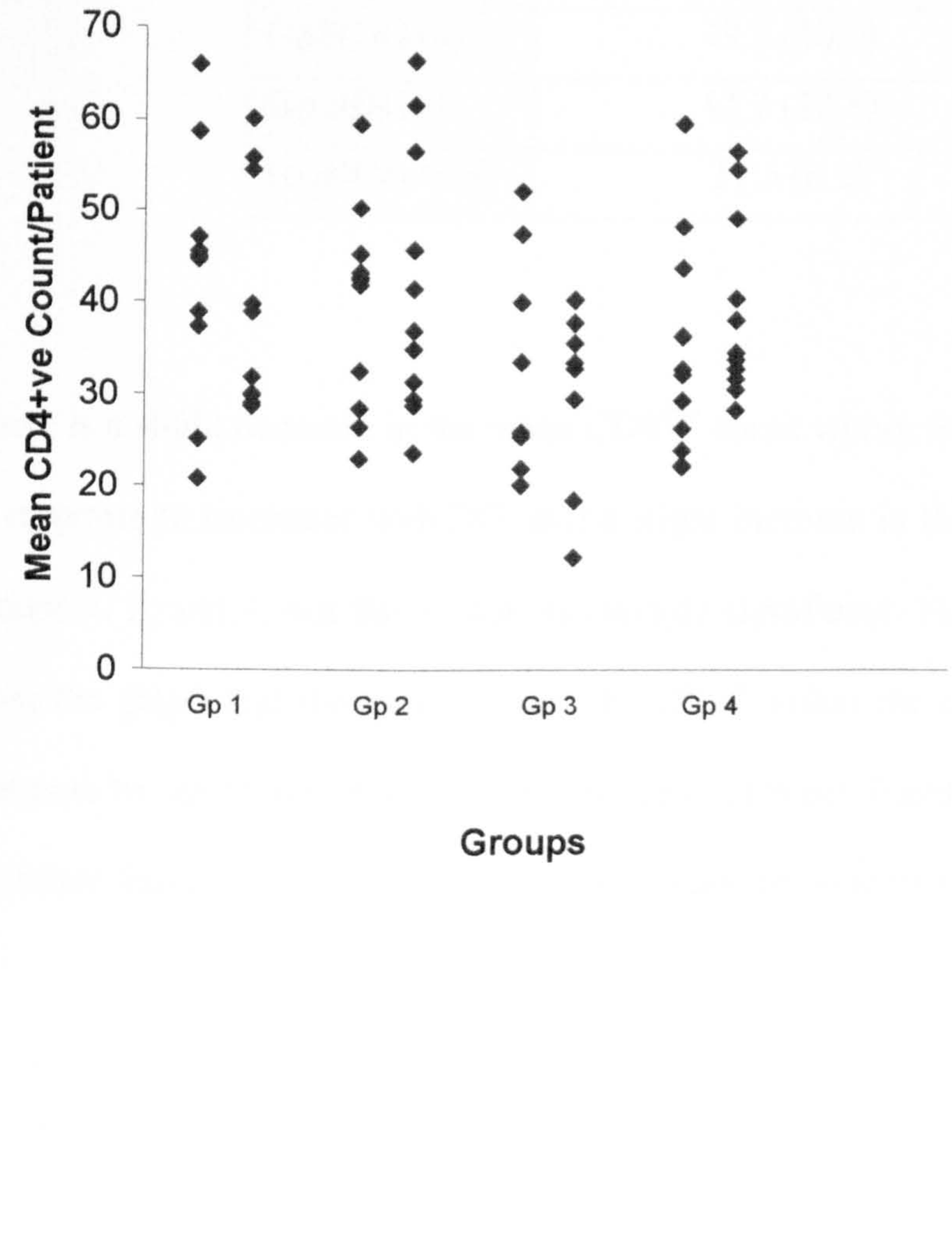
Groups	Week 0 Mean (SD)
Gp1(DGx12)	42.9 (13.6)
Gp2(DGx4)	39.6 (10.9)
Gp3(DGx1)	33.3 (12.0)
Gp4(Control)	34.2 (11.9)

There is a slight decrease in the mean $CD4^{+ve}$ population within the wound bed of groups 1 and 3 in response to treatment with DG, and a slight increase in the mean $CD4^{+ve}$ population in groups 2 and 4, but this is not statistically significant. (Table 4-29). This is also seen graphically in Figure 4-15.

Table 4-28 The mean count of $CD4^{+ve}$ helper/inducer T-Lymphocytes within chronic wounds at Week 0 and Week 6

Groups	Week 0 Mean (SD)	Week 6 Mean (SD)	Mann – Whitney U P values
Gp1(DGx12)	42.9 (13.6)	39.9 (12.40	0.5
Gp2(DGx4)	39.6 (10.9)	41.5 (14.3)	0.9
Gp3(DGx1)	33.3 (12.0)	30.0 (9.70	0.72
Gp4(Control)	34.2 (11.9)	39.1 (9.8)	0.2

Figure 4-15 Mean CD4+ve helper/inducer T-Lymphocytes Counts in Chronic Wounds at Week 0 and Week 6 for the Four Groups



There is little change in the mean count of $CD4^{+ve}$ cells at week 6 when compared to week 0

The T suppressor/cytotoxic lymphocytes are identified by the positive staining of cells within the wound bed tissue with the anti-CD8 monoclonal antibody. Staining with this antibody demonstrates that there was little difference in the counts between the four groups atWeek 0. (Table 4-29)

Table 4-29 The mean count of $CD8^{+ve}$ suppressor/cytotoxic T-lymphocytes at Week 0 within the four groups

Groups	Week 0 Mean (SD)
Gp1(DGx12)	18.4 (10.4)
Gp2(DGx4)	19.2 (10.0)
Gp3(DGx1)	11.2 (10.8)
Gp4(Control)	11.5 (6.1)

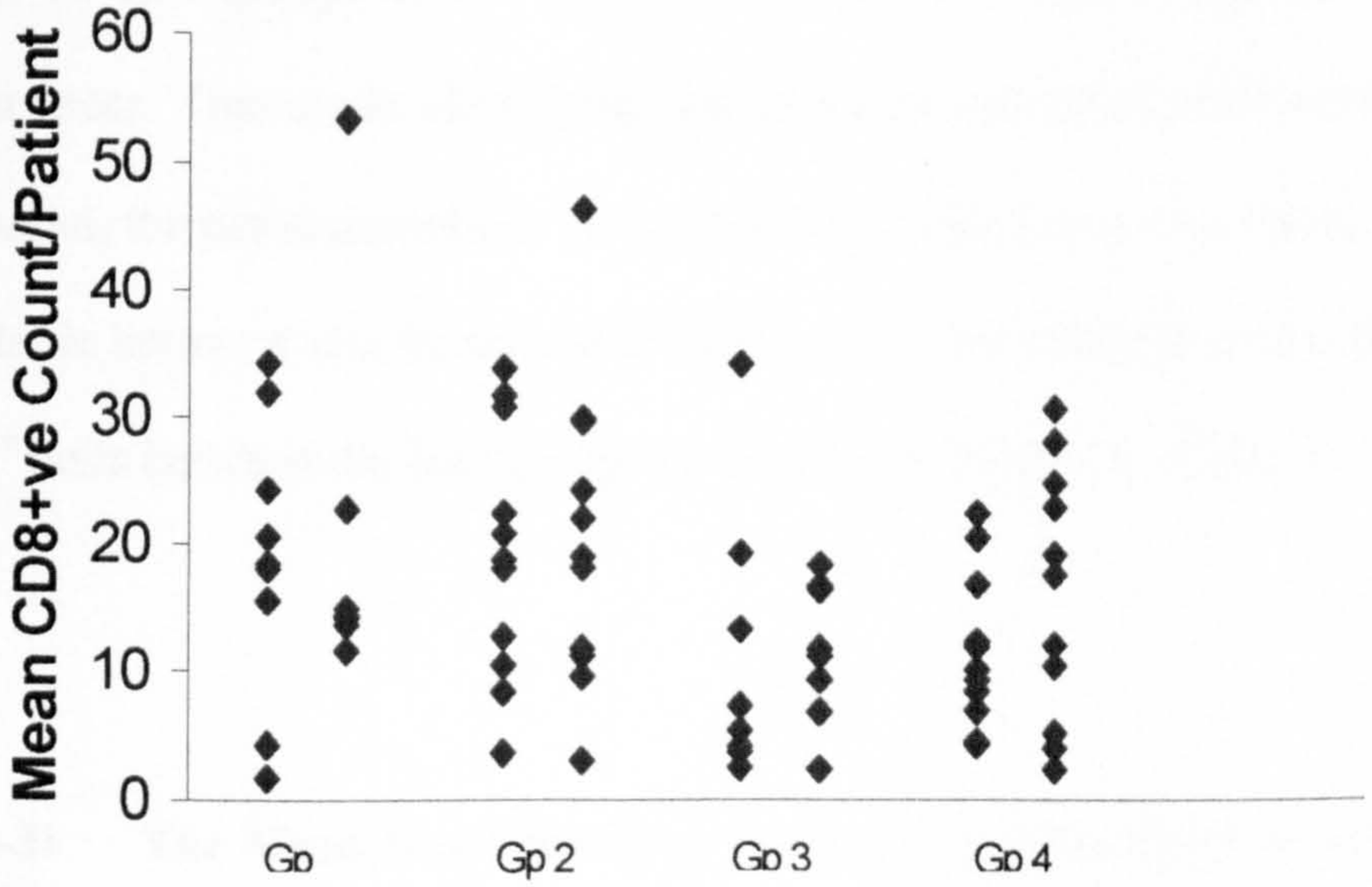
There is a slight decrease in the mean $CD8^{+ve}$ count within the wound bed of group 3 in response to treatment with DG, and a slight increase in the mean $CD8^{+ve}$ count in groups 1, 2, and 4, but this is not statistically significant. However one can observe from the graph that the mean values of $CD8^{+ve}$ within the control group (Group 4) seems to be tightly orientated around the mean at Week 0 and Week 6, and hence can postulate that compression alone in these groups of patients causes an increase in the

mean counts at week 6, an observation not apparent in the treatment groups. (Table 4-30 and Figure 4-16)

Table 4-30 The mean count of $CD8^{+ve}$ T-Lymphocytes within chronic wounds at Week 0 and Week 6

Groups	Week 0 Mean (SD)	Week 6 Mean (SD)	Mann – Whitney U P values
Gp1(DGx12)	18.4 (10.4)	18.2 (12.8)	0.3
Gp2(DGx4)	19.2 (10.0)	20.4 (12.1)	0.65
Gp3(DGx1)	11.2 (10.8)	11.5 (5.4)	0.79
Gp4(Control)	11.5 (6.1)	16.1 (9.9)	0.32

Figure 4-16 Mean $CD8^{+ve}$ Counts in Chronic Wounds at Week 0 and Week 6 for the Four Groups.



There is a slight increase in the mean $CD8^{+ve}$ count in groups 1, 2, and 4, but this is not statistically significant

4.2.9.1.2 *Macrophages*

Cells exhibiting macrophage morphology and staining with the pan-macrophage marker anti-CD68 monoclonal antibody were found throughout the wound bed sections in all four groups at both weeks 0 and 6, with dense aggregations in the perivasular areas. This made identifying and counting individual cells very difficult. For this reason, the pan-macrophage expression was graded on a 1 to 4 basis.

From the table below, it can be seen that there is very little change in the distribution of $CD68^{+ve}$ cells between the four groups at Week 0 and Week 6. (Table 4-31)

Table 4-31 The Mean Score of $CD68^{+ve}$ cells in Chronic Wounds at Week 0 and Week 6

Groups	Week 0 Mean Score	Week 6 Mean Score
Gp1(DGx12)	4	4
Gp2(DGx4)	4	4
Gp3(DGx1)	3.5	3.5
Gp4(Control)	4	4

When the trend of change at week 6 was compared to week 0 for each patient in each group, it can be seen that there is very little change in the mean score of $CD68^{+ve}$ cells in the two time frames. (Table 4-32)

Table 4-32 Comparison of CD68 in Chronic Wounds staining intensity at Week 6 to Week 0.

Groups	Change in the Intensity of Staining at Week 6 when Compared to Week 0		
	Increase	**Decrease**	**Unchanged**
Gp1(DGx12)	3	2	5
Gp2(DGx4)	1	3	7
Gp3(DGx1)	1	1	6
Gp4(Control)	2	4	4

Subpopulations of Macrophages

27e10

The 27e10 antigen is usually found as a surface marker on early macrophages. These macrophages are often found in abundance in acutely inflamed tissue but occur in small numbers in chronic wounds.

There is little difference between groups 2, 3, and 4 for the $27e10^{+ve}$ macrophage markers at week 0. (Table 4-33) However, there seems to be a statistically significant number of $27e10^{+ve}$ macrophages in group 1 when compared to the control group ($p=0.03$). This may not be a true significance but may be due to chance as the number of patients in each group is small and not a true reflection of an unexplained increase of early macrophages within this group of patients.

Table 4-33 The mean count of $27e10^{+ve}$ macrophages at Week 0 within the four groups

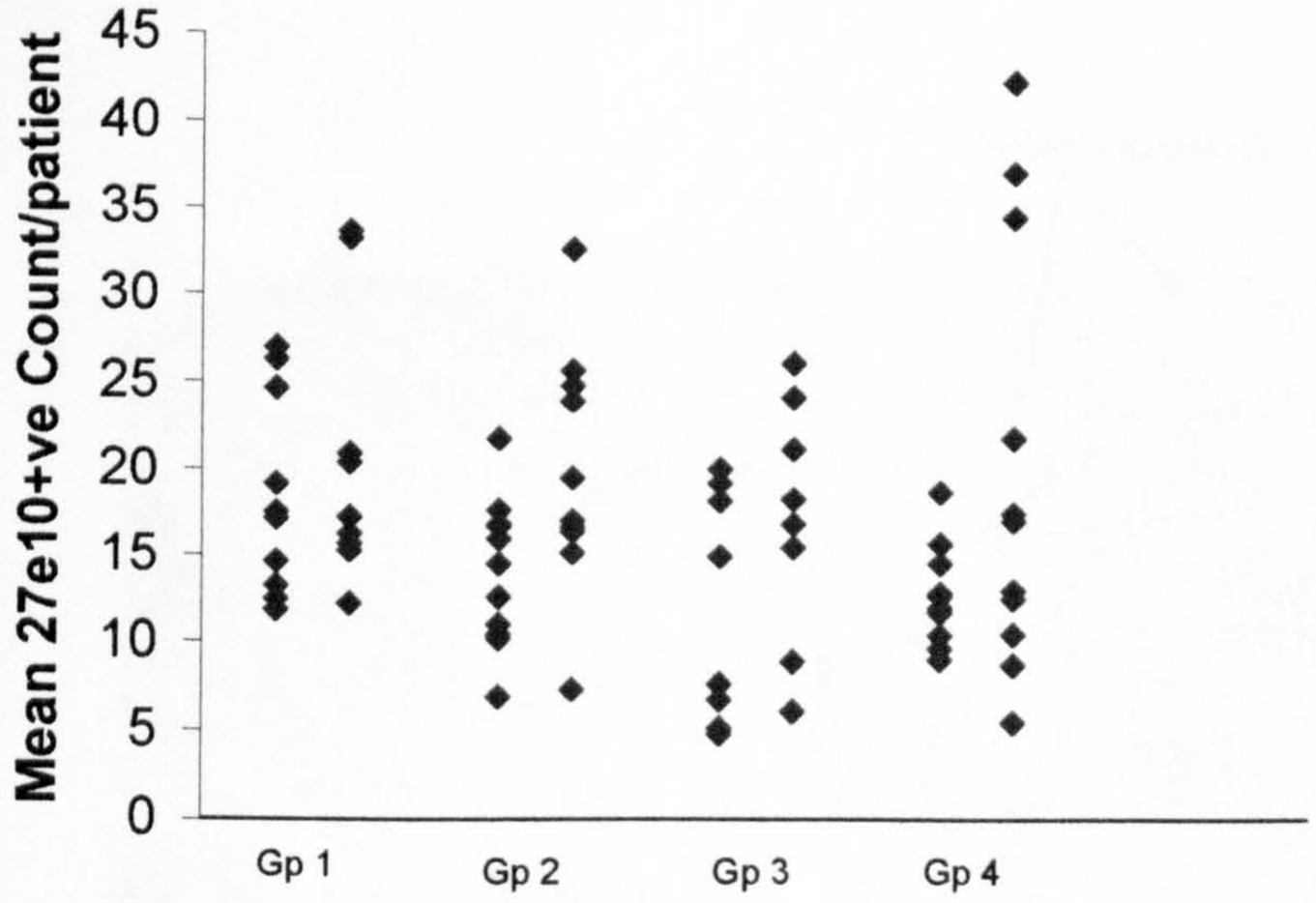
Groups	Week 0 Mean (SD)
Gp1(DGx12)	18.4 (5.6)
Gp2(DGx4)	14.0 (4.2)
Gp3(DGx1)	12.0 (6.6)
Gp4(Control)	13.3 (3.5)

There seems to be an increase in the mean count of early macrophages at week 6 when compared to week 0 in all groups, and this increase is statistically significant in group 2 ($p<0.05$). (Table. 4-34, Figures.4-17, 4-18)

Table 4-34 The mean count of $27e10^{+ve}$ macrophages within chronic wounds at Week 0 and Week 6

Groups	Week 0 Mean (SD)	Week 6 Mean (SD)	Mann – Whitney U P values
Gp1(DGx12)	18.4 (5.6)	20.2 (7.4)	0.63
Gp2(DGx4)	14.0 (4.2)	21.0 (7.7)	0.02
Gp3(DGx1)	12.0 (6.6)	17.1 (7.0)	0.16
Gp4(Control)	13.3 (3.5)	20.0 (12.4)	0.3

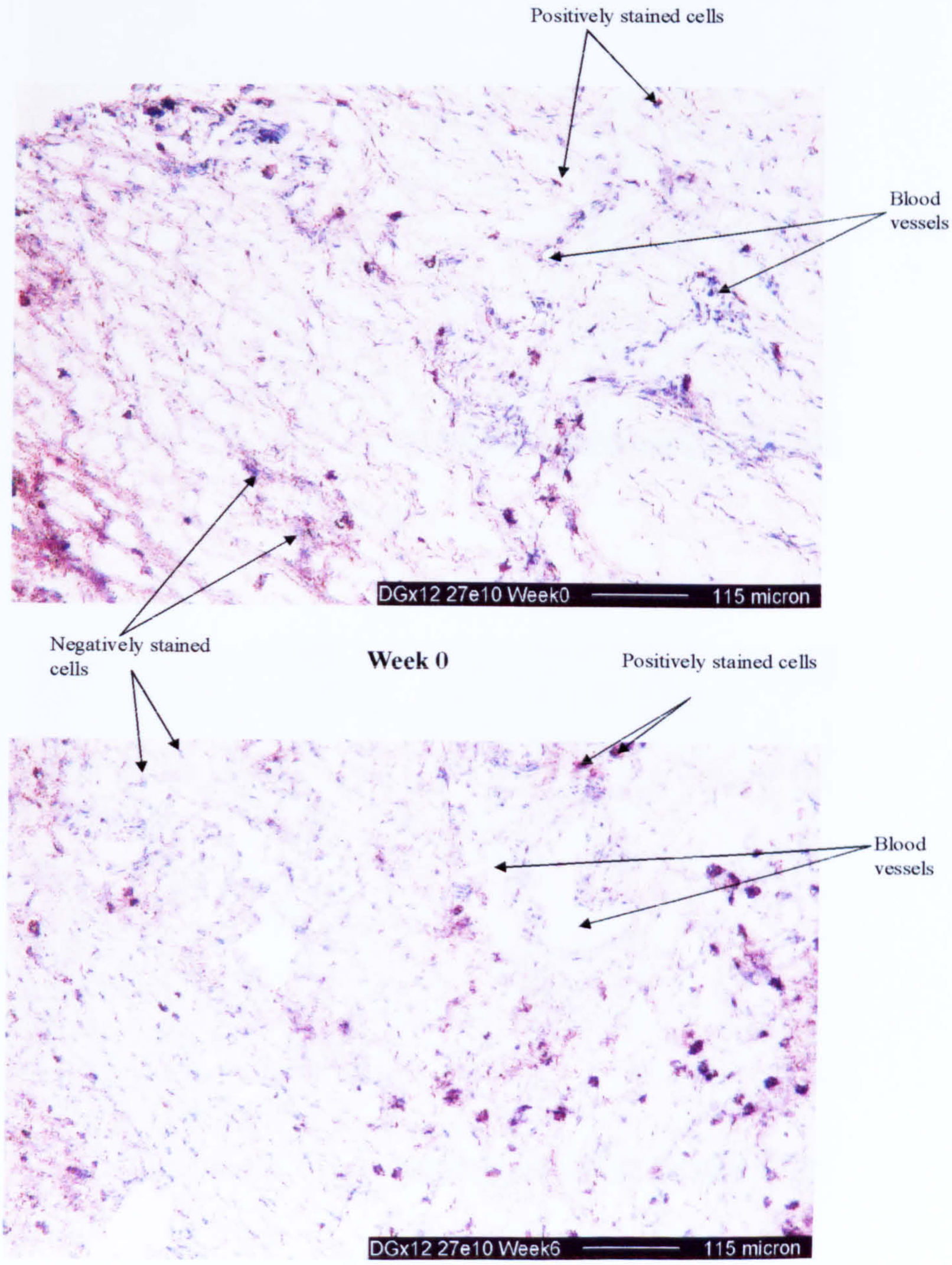
Figure 4-17 Mean 27e10^{+ve} Counts in Chronic Wounds at Week 0 and Week 6 for the Four Groups.



There seems to be an increase in the mean count of early macrophages at week 6 when compared to week 0 in all groups, and this increase is statistically significant in group 2

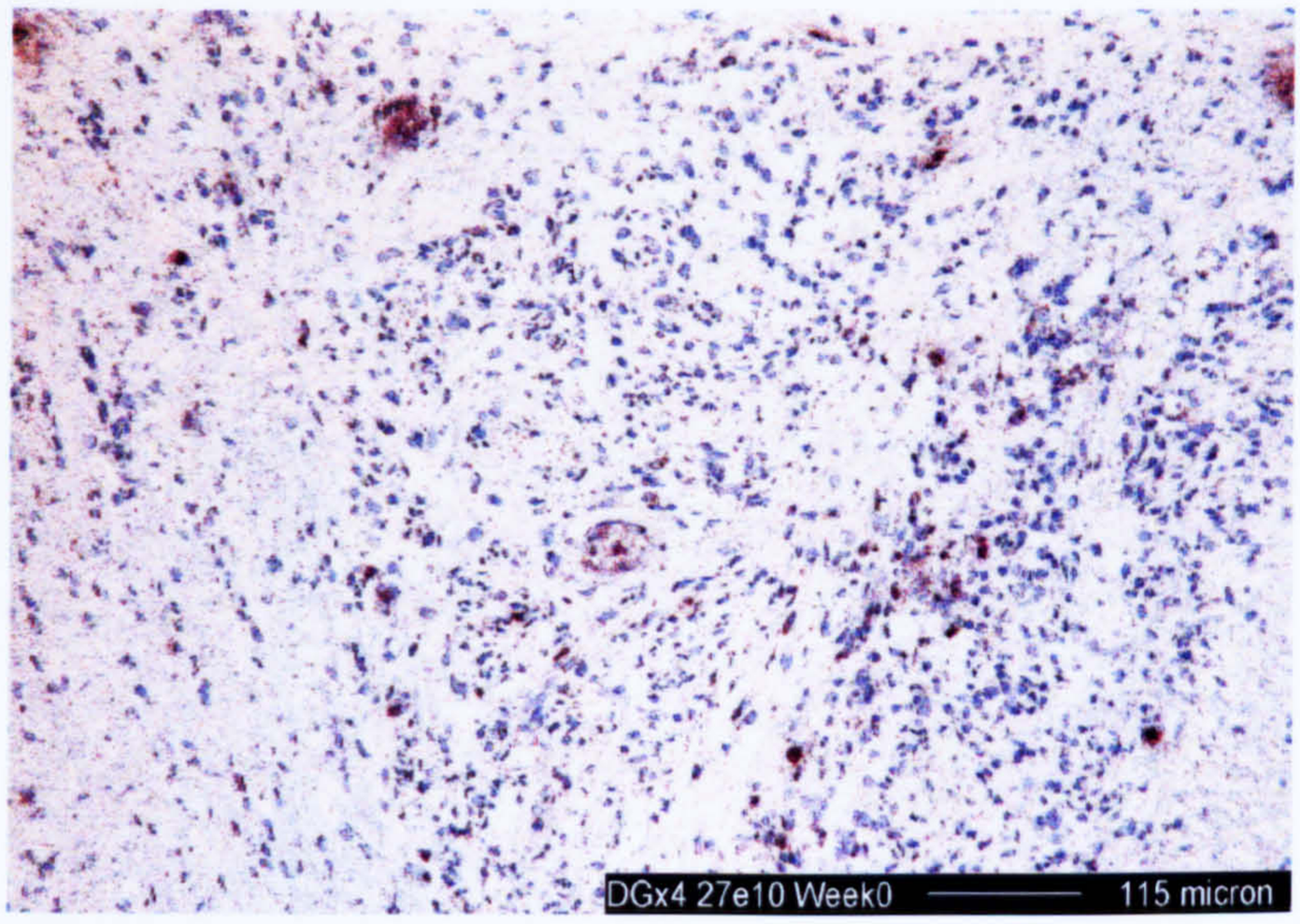
Figure 4-18 Photomicrograph of 27e10^{+ve} cells at Week 0 and Week 6in the Four Treatment Groups

Group 1 (DGx12) – There is an increase in the number of early macrophages at week 6 compared to week 0

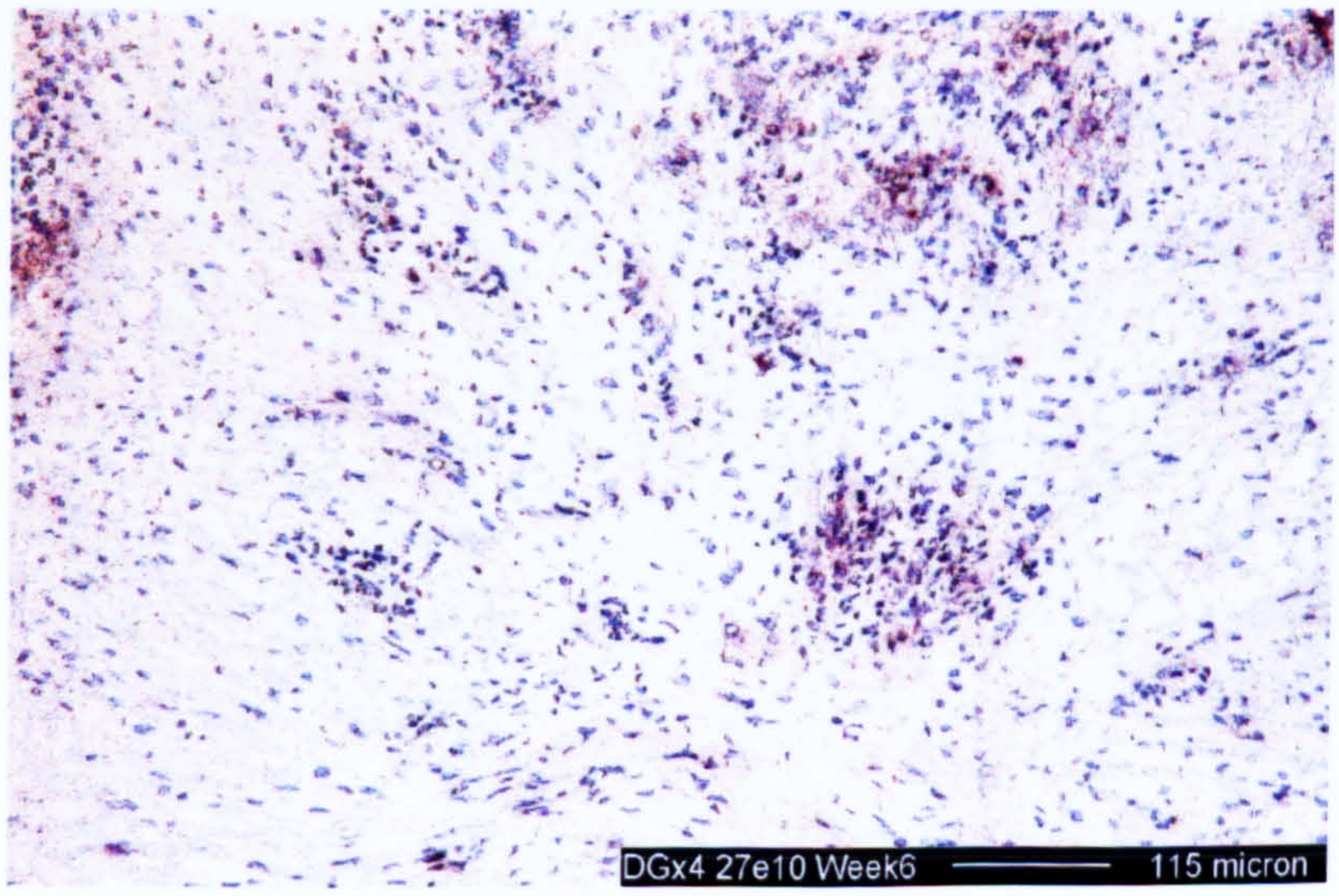


Week 6

Group 2 (DGx4) – There is an increase in the number of early macrophages at week 6 compared to week 0 which is statistically significant

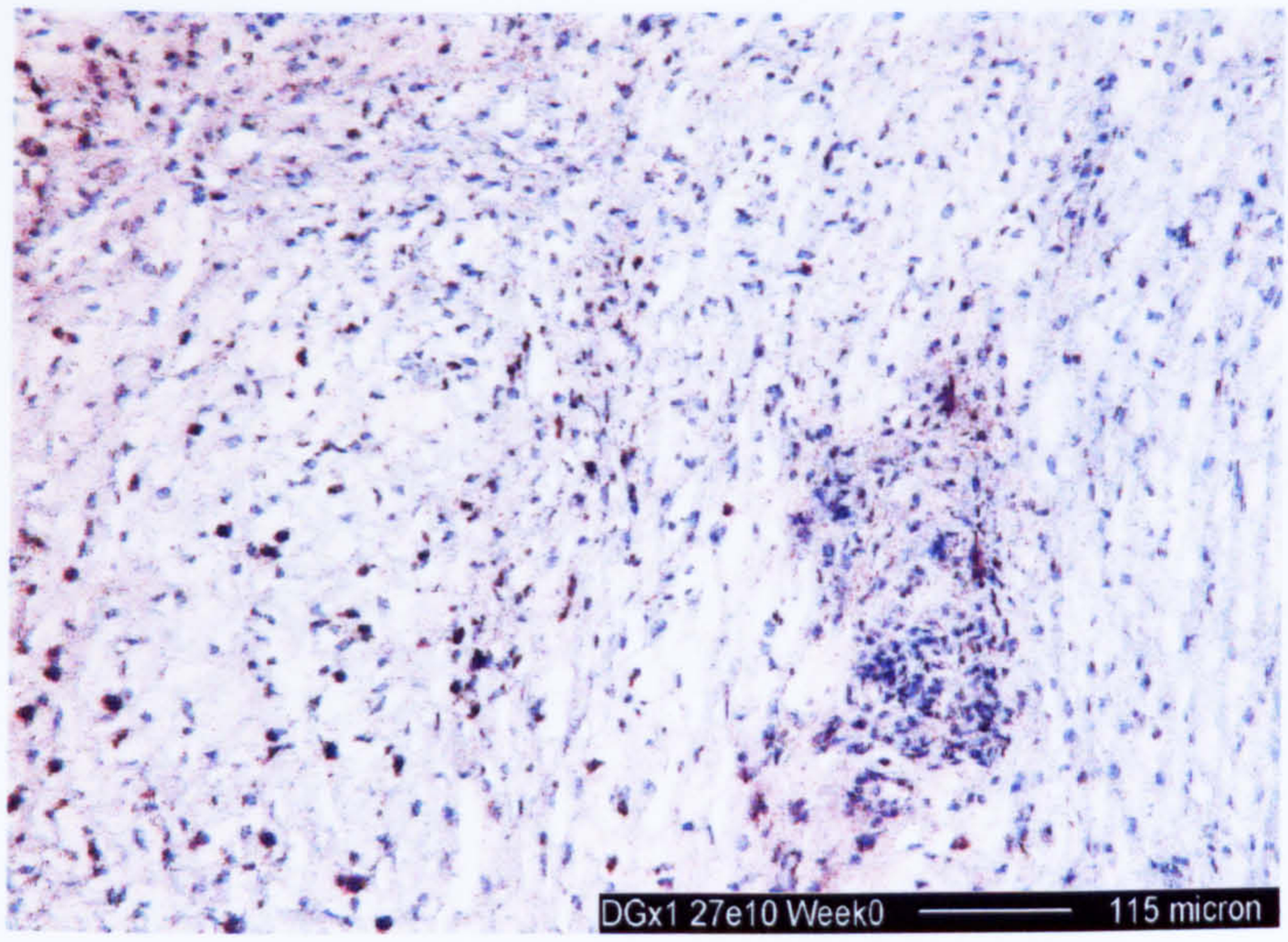


Week 0

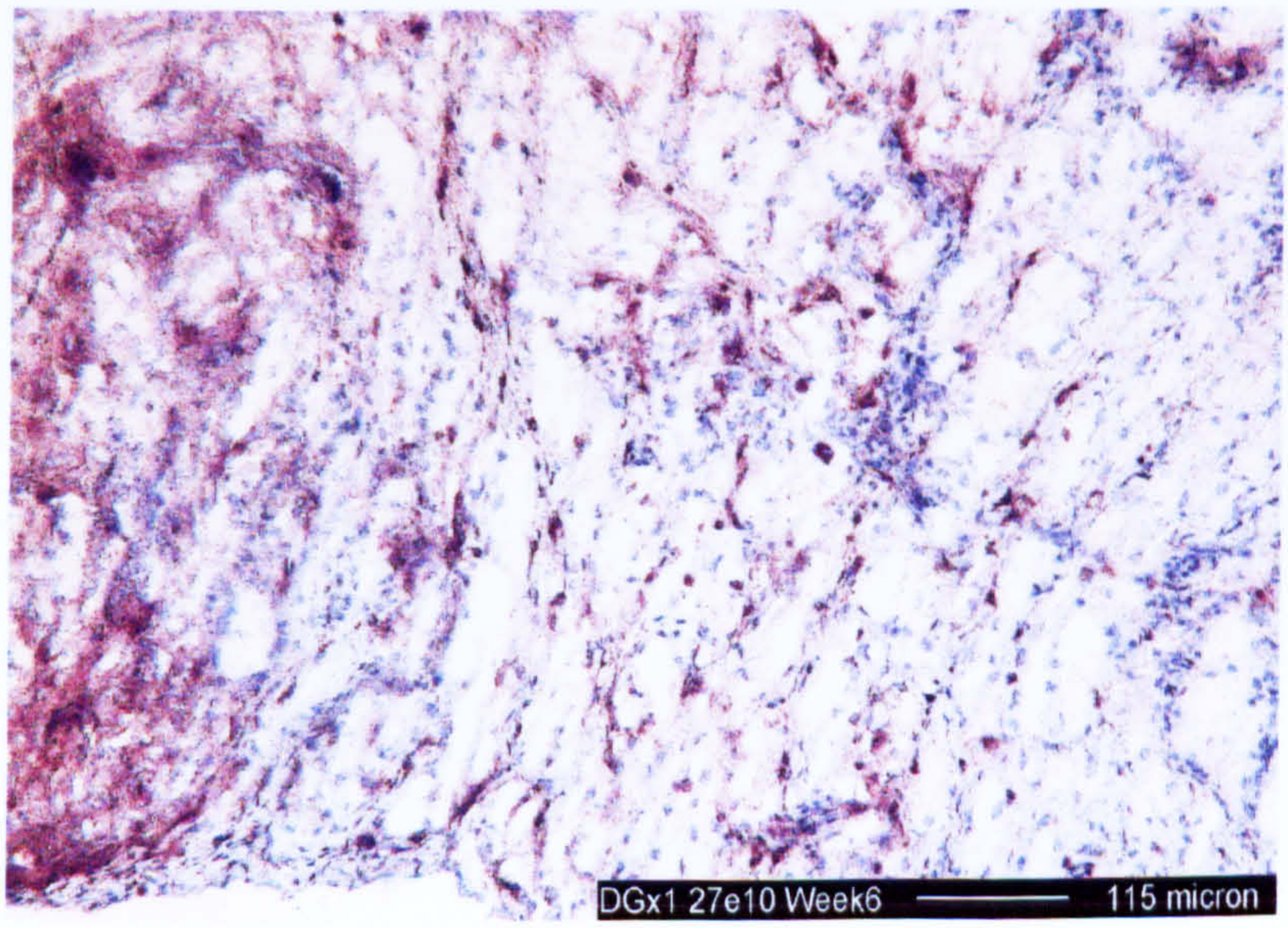


Week 6

Group 3 (DGx1) - There is an increase in the number of early macrophages at week 6 compared to week 0

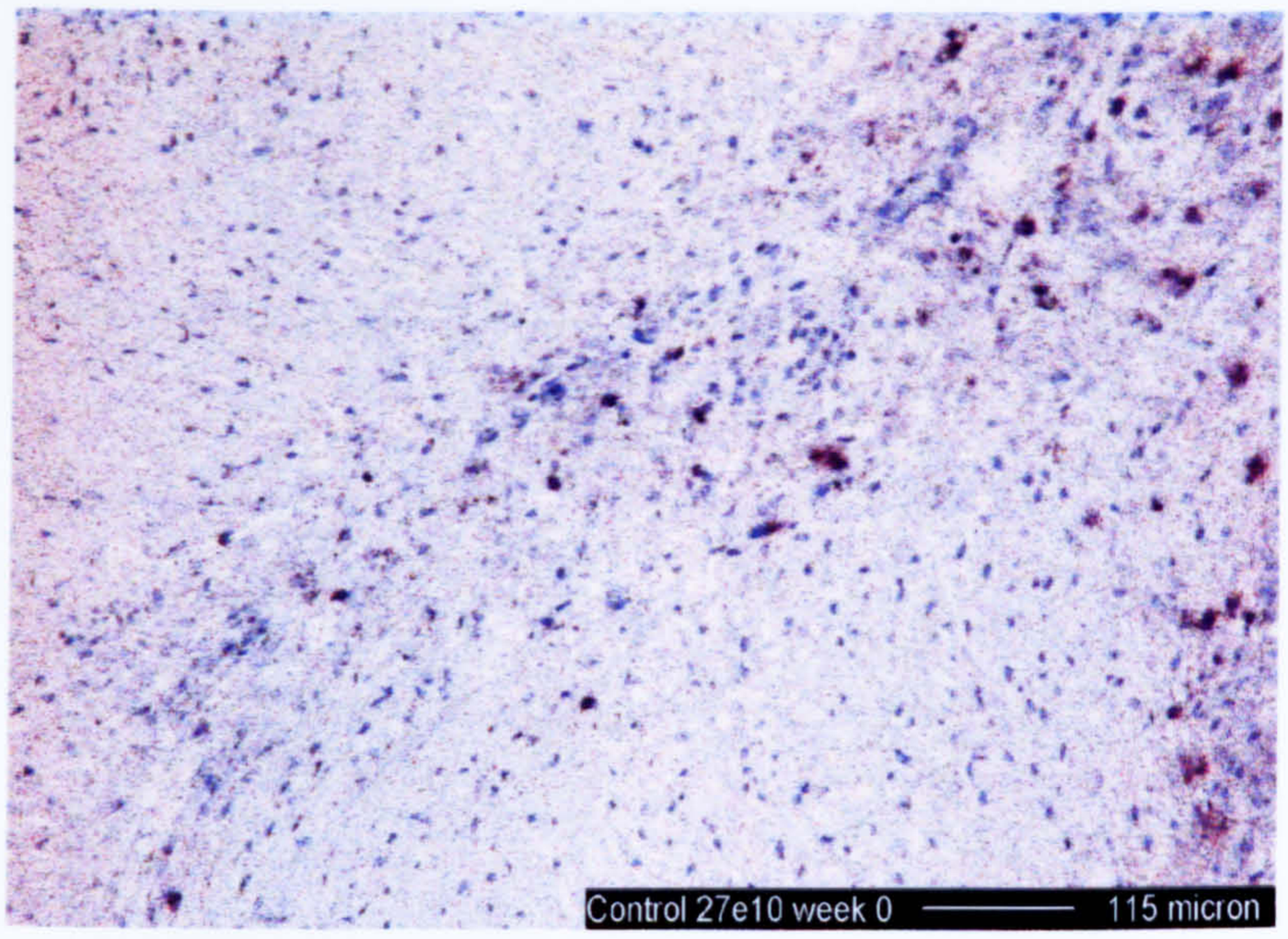


Week 0

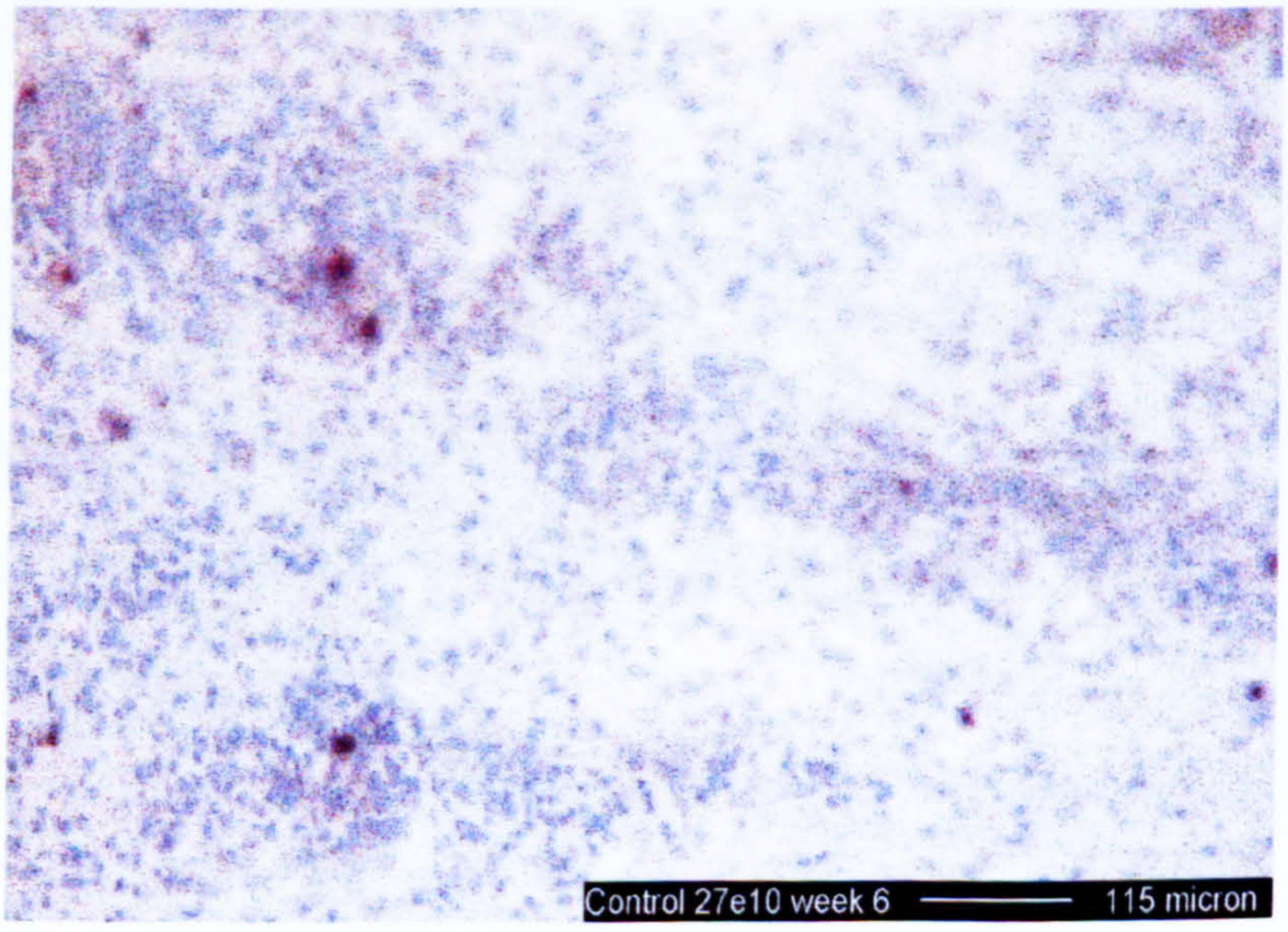


Week 6

Group 4 (Control) – There is relatively little change in the number of early macrophages at week 6 compared to week 0



Week 0



Week 6

Mannose Receptors (MR)

The mannose receptor is expressed on the surface of primed macrophages, which subsequently disappears when these macrophages become activated.

There is little difference between the four treatment groups for the mean count of MR^{+ve} cells at week 0. (Table 4-35).

Table 4-35 The mean count of MR^{+ve} macrophages at Week 0 within the four groups

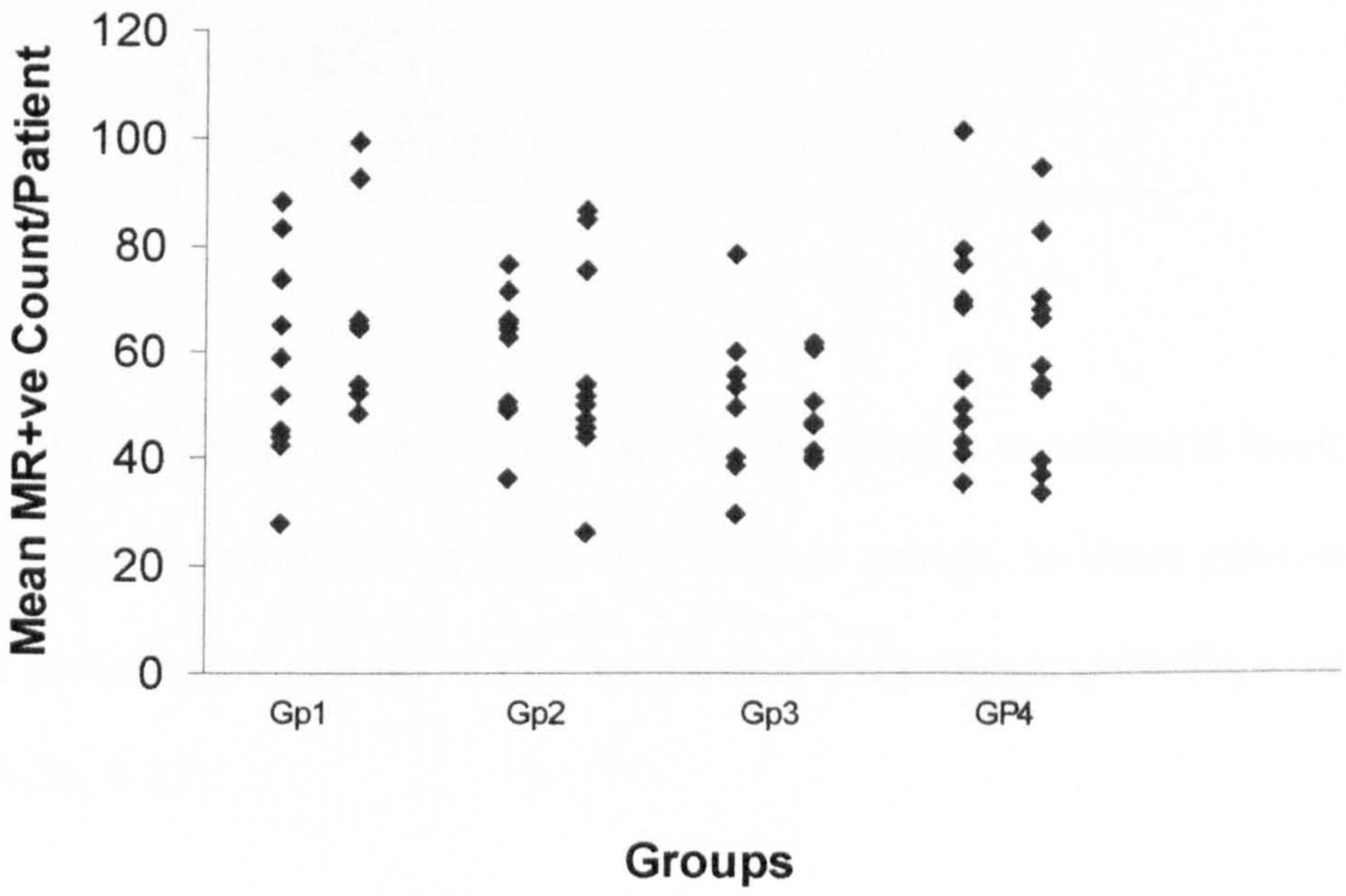
Groups	Week 0 Mean (SD)
Gp1(DGx12)	57.9 (19.6)
Gp2(DGx4)	57.9 (12.7)
Gp3(DGx1)	50.5 (15.1)
Gp4(Control)	60.2 (20.0)

There is no significant change in the mean count of MR^{+ve} macrophages at week 6 when compared to week 0. There seems to be a slight increase in the mean MR^{+ve} count at week 6 when compared to week 0 in group 1, but this may simply be a variation rather than a true reflection due to the small number of patients within the group. (Table 4-36, Figure 4-19)

Table 4-36 The mean count of MR^{+ve} macrophages within chronic wounds at Week 0 and Week 6

Groups	Week 0 Mean (SD)	Week 6 Mean (SD)	Mann – Whitney U P values
Gp1(DGx12)	57.9 (19.6)	64.6 (17.8)	0.35
Gp2(DGx4)	57.9 (12.7)	55.4 (18.9)	0.75
Gp3(DGx1)	50.5 (15.1)	48.3 (8.8)	0.9
Gp4(Control)	60.2 (20.0)	59.5 (19.0)	0.9

Figure 4-19 Mean MR^{+ve} Counts in Chronic Wounds at Week 0 and Week 6 for the Four Groups



The distribution of MR^{+ve} cells seems to be similar in all four treatment groups

Sialoadhesin Receptors (SR)

Sialoadhesin receptors are surface antigens expressed by tissue macrophages. They are believed to be the result of those primed macrophages that remain unactivated within the wound bed.

There is little difference between the four treatment groups for the SR^{+ve} macrophage markers at week 0 (Table 4-37).

Table 4-37 The mean count of SR^{+ve} macrophages at Week 0 within the four groups

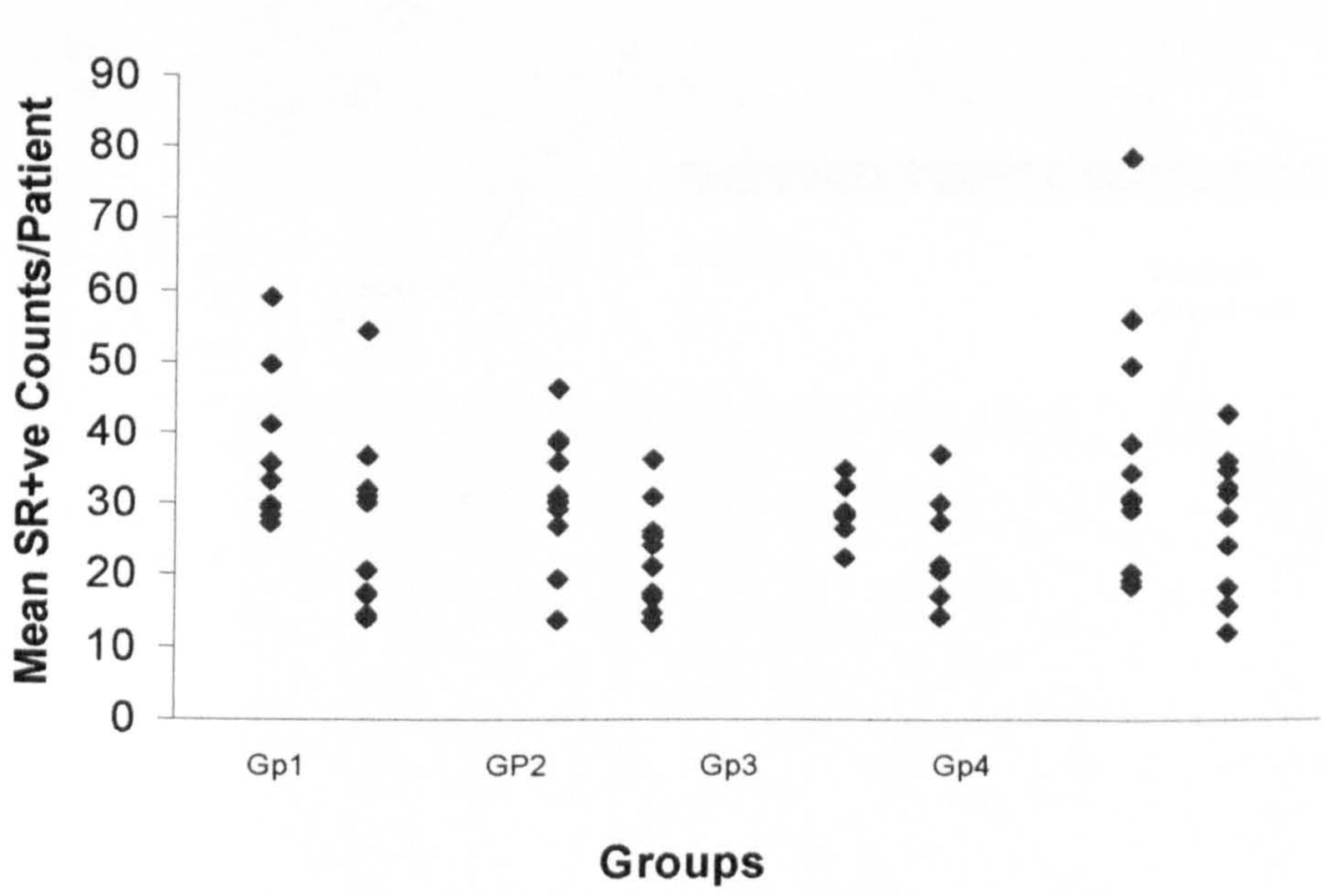
Groups	Week 0 Mean (SD)
Gp1(DGx12)	36.9 (10.4)
Gp2(DGx4)	31.0 (9.2)
Gp3(DGx1)	28.8 (3.8)
Gp4(Control)	36.8 (18.2)

There is a trend for the mean count of SR^{+ve} macrophages to reduce at week 6 when compared to the mean count at week 0 in all four groups. In those patients treated with four pieces of DG (group 2), this is statistically significant ($p<0.05$). (Table 4-38, Figures 4-20, 4-21)

Table 4-38 The mean count of SR^{+ve} macrophages within chronic wounds at Week 0 and Week 6

Groups	Week 0 Mean (SD)	Week 6 Mean (SD)	Mann – Whitney U P values
Gp1(DGx12)	36.9 (10.4)	26.8 (12.7)	0.11
Gp2(DGx4)	31.0 (9.2)	22.8 (7.0)	0.03
Gp3(DGx1)	28.8 (3.8)	23.5 (7.50	0.10
Gp4(Control)	36.8 (18.2)	26.5 (10.1)	0.20

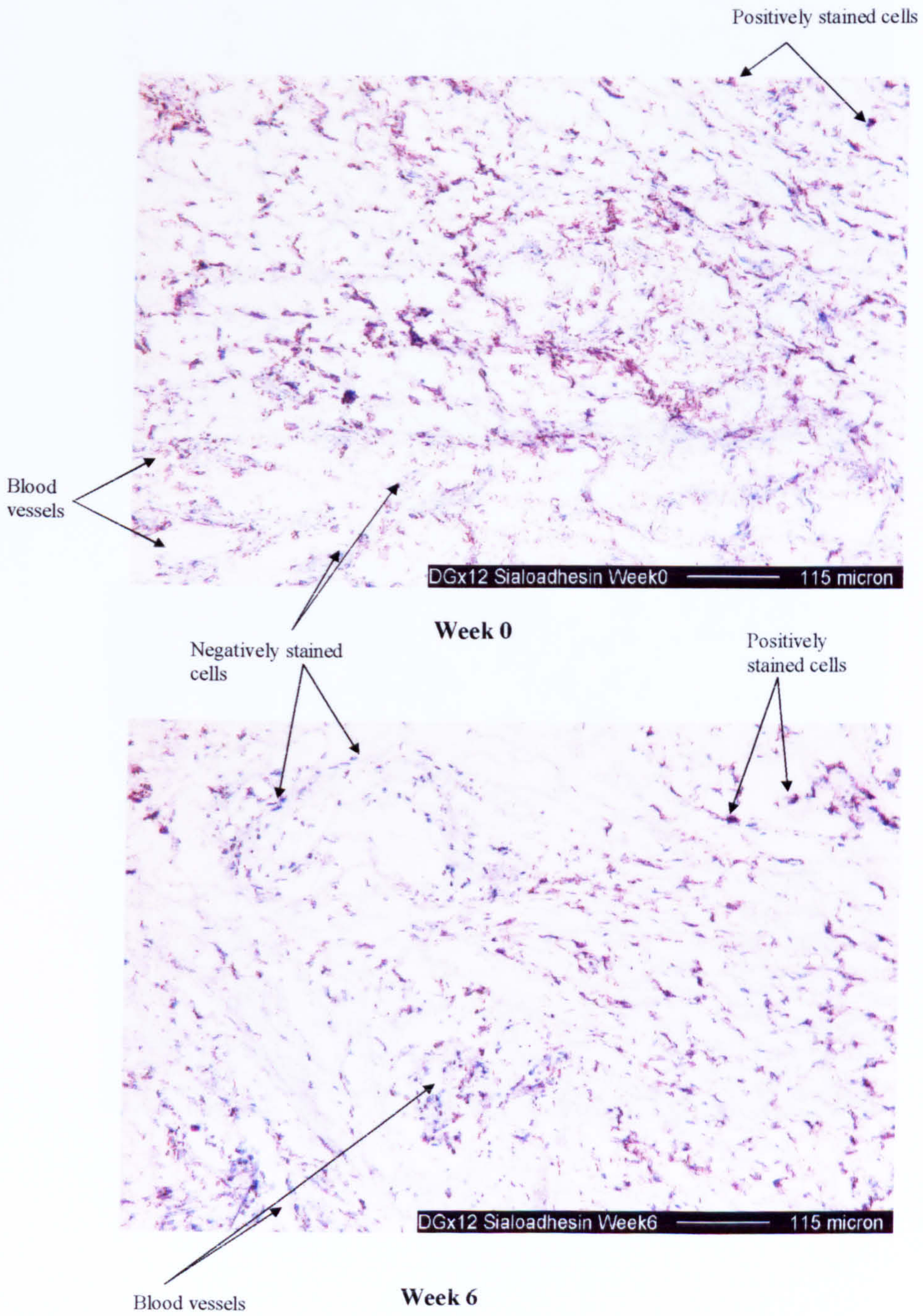
Figure 4-20 Mean SR^{+ve} Counts in Chronic Wounds at Week 0 and Week 6 for the Four Groups



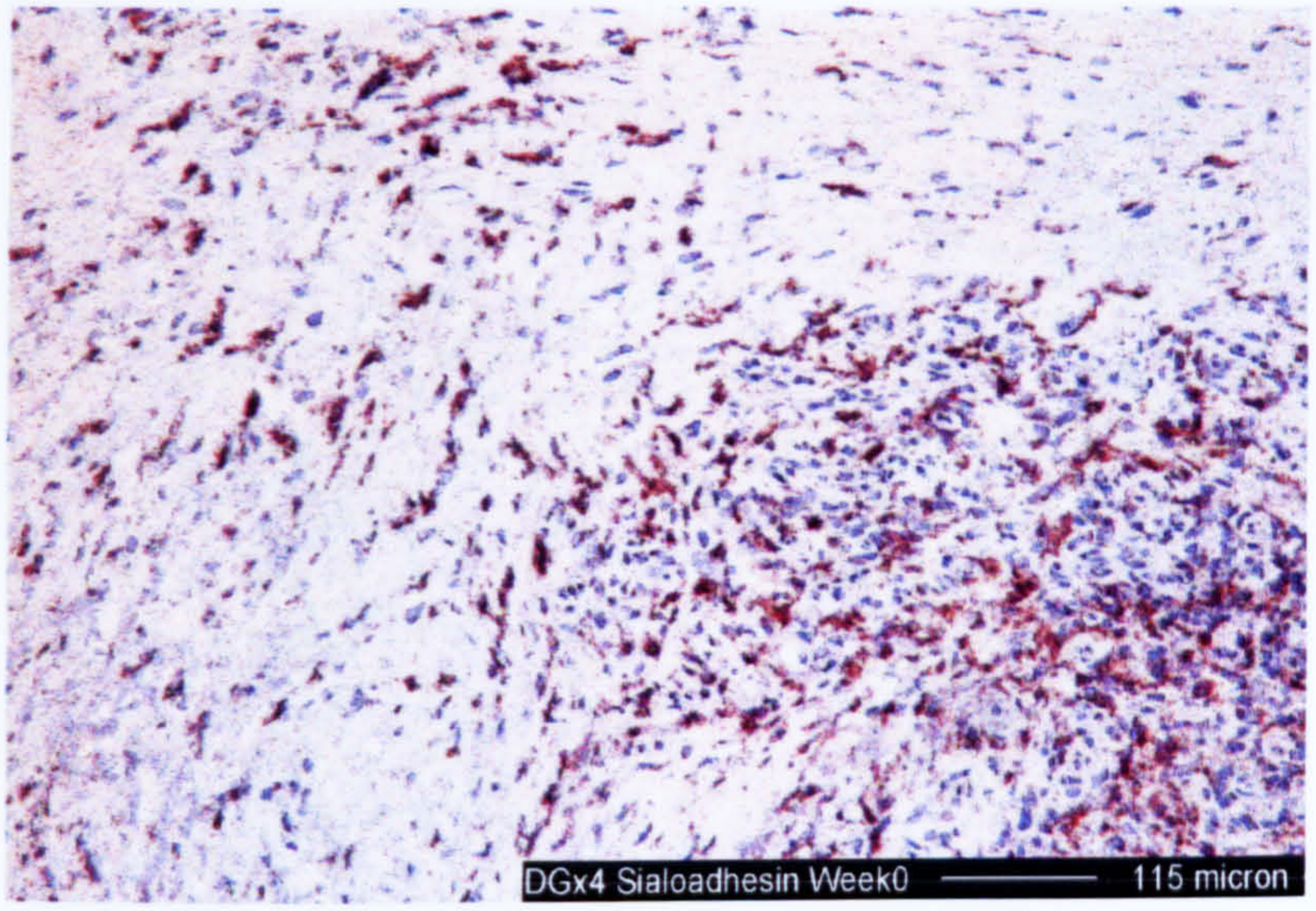
The mean count of SR^{+ve} macrophages to reduce at week 6 when compared to the mean count at week 0 in all four groups, and is statistically significant in group 2

Figure 4-21 Photomicrographs of SR^{+ve} Cells at Week 0 and Week 6 in the Four Treatment Groups

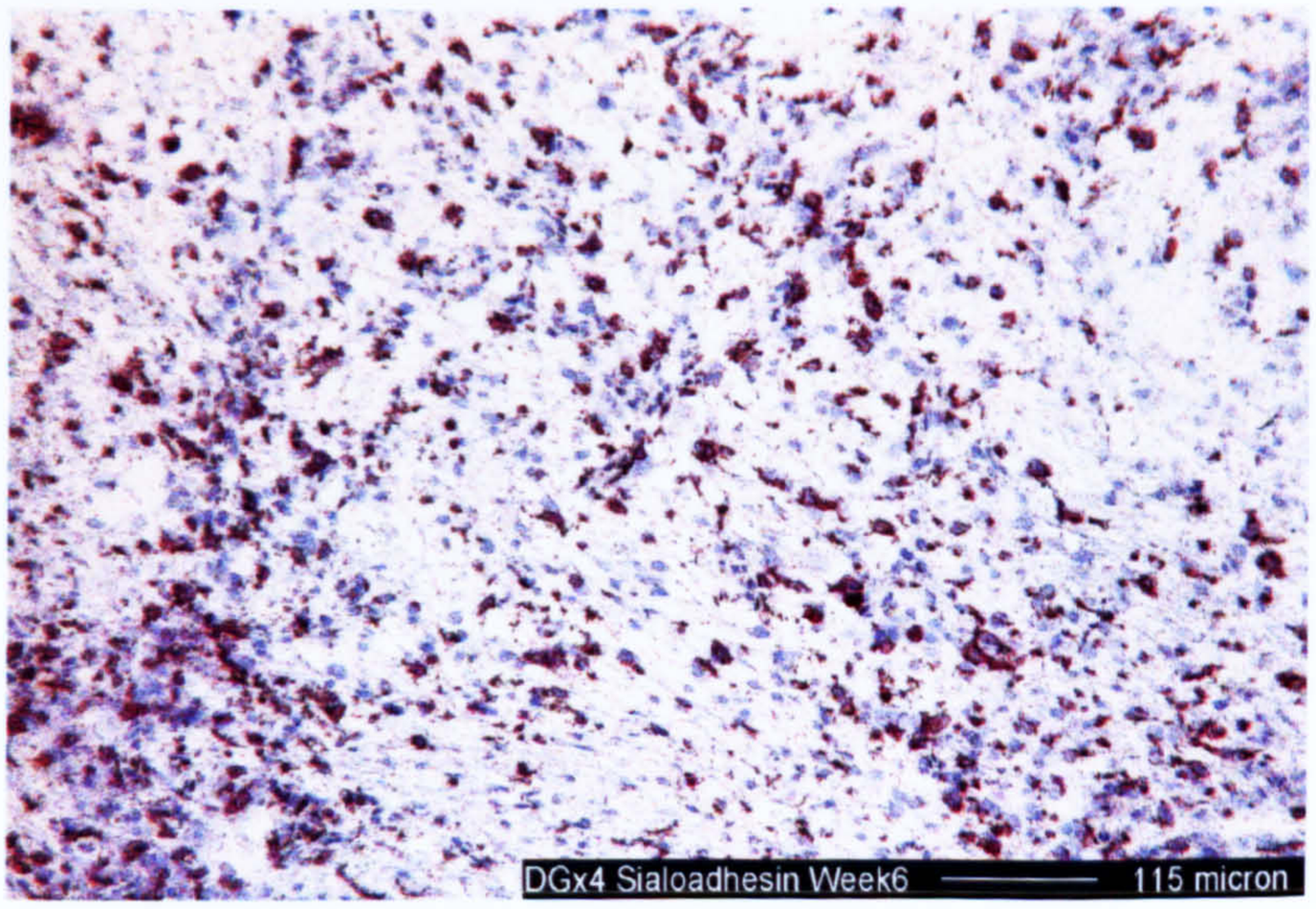
Group 1 (DGx12) – There is a reduction in the number of tissue macrophages at week 6 when compared to week 0



Group 2 (DGx4) - There is a reduction of tissue macrophages at week 6 when compared to week 0

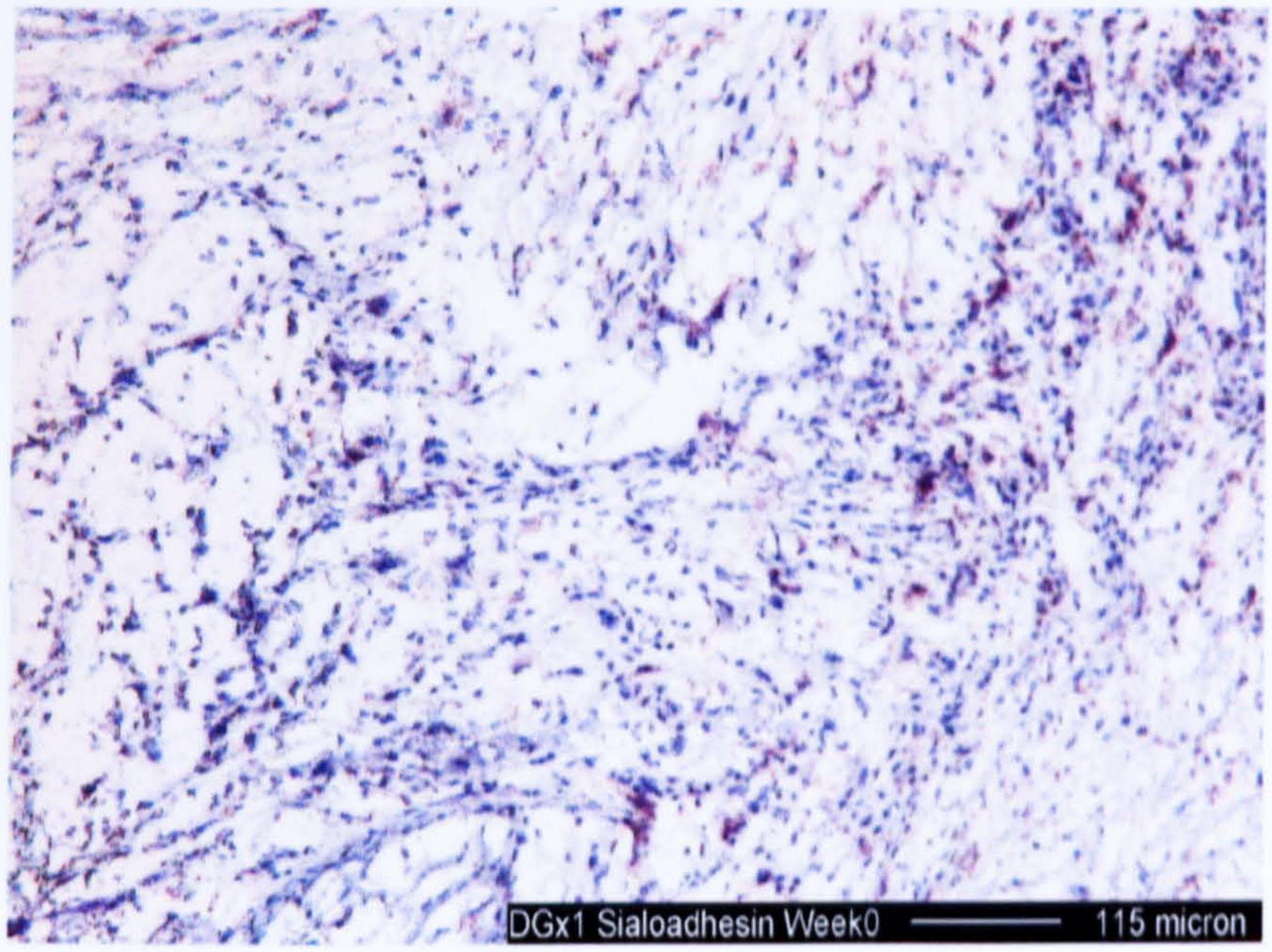


Week 0

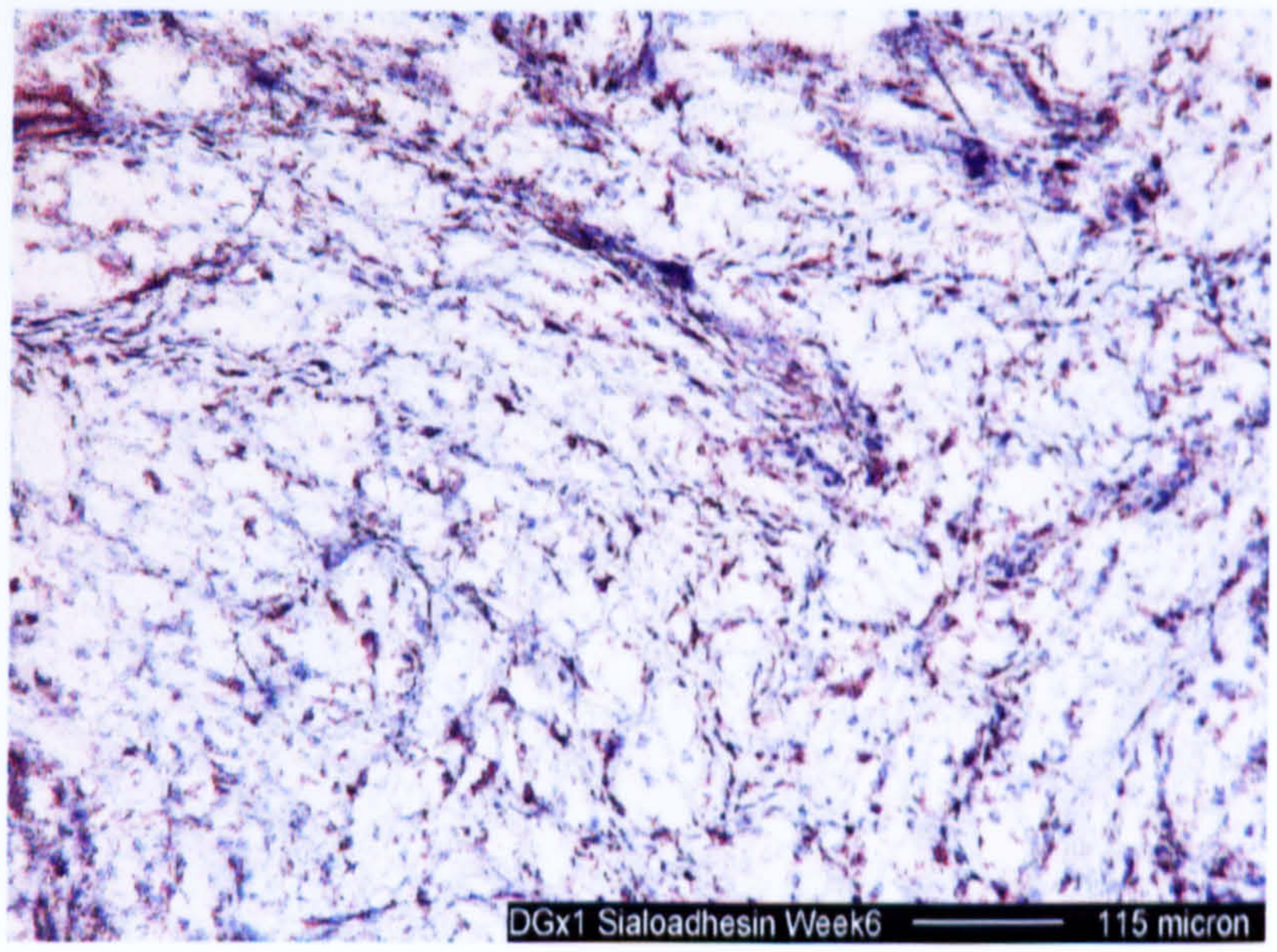


Week 6

Group 3 (DGx1) – There is little difference in the number of tissue macrophages at week 6 when compared to week 0

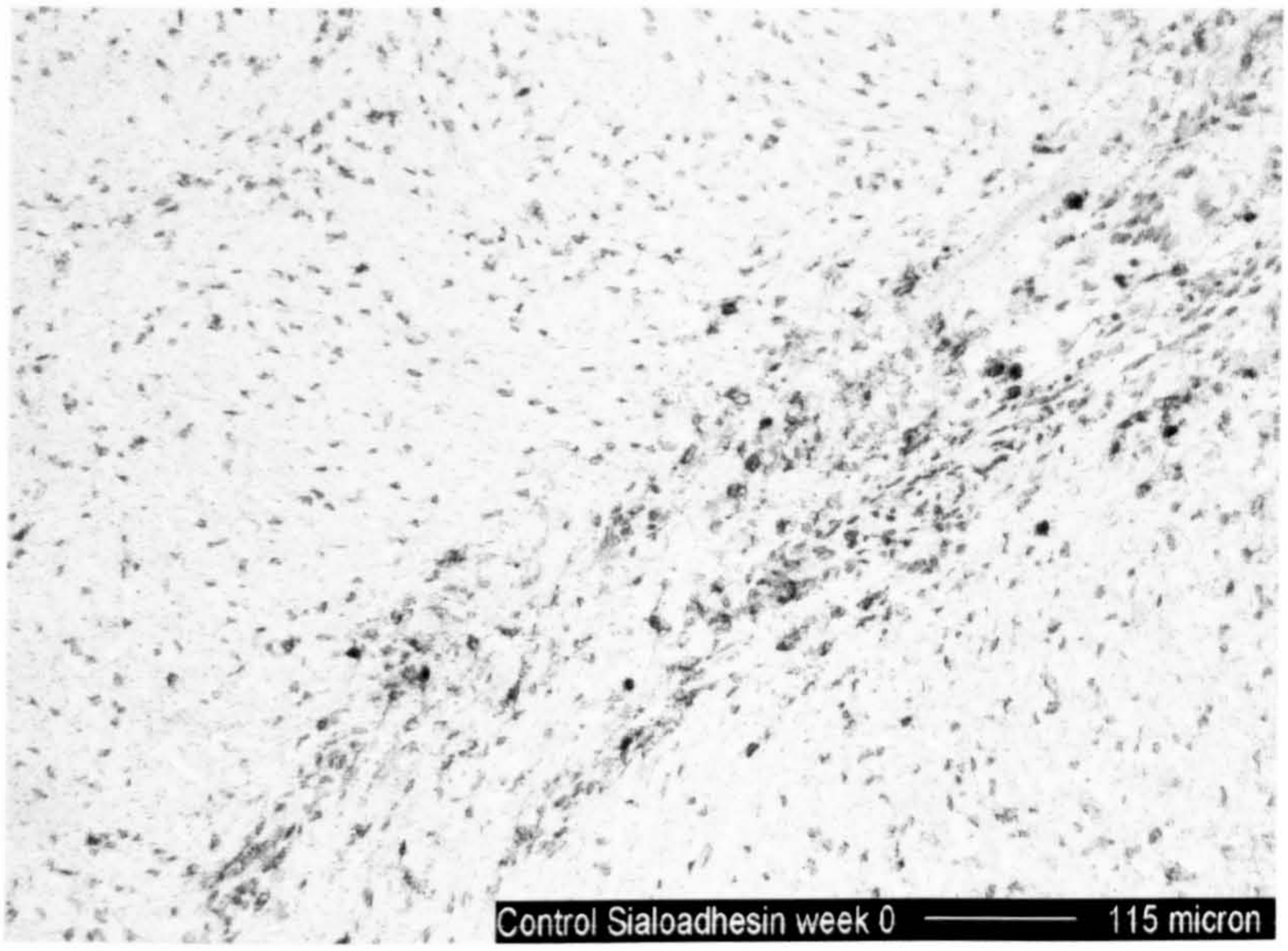


Week 0

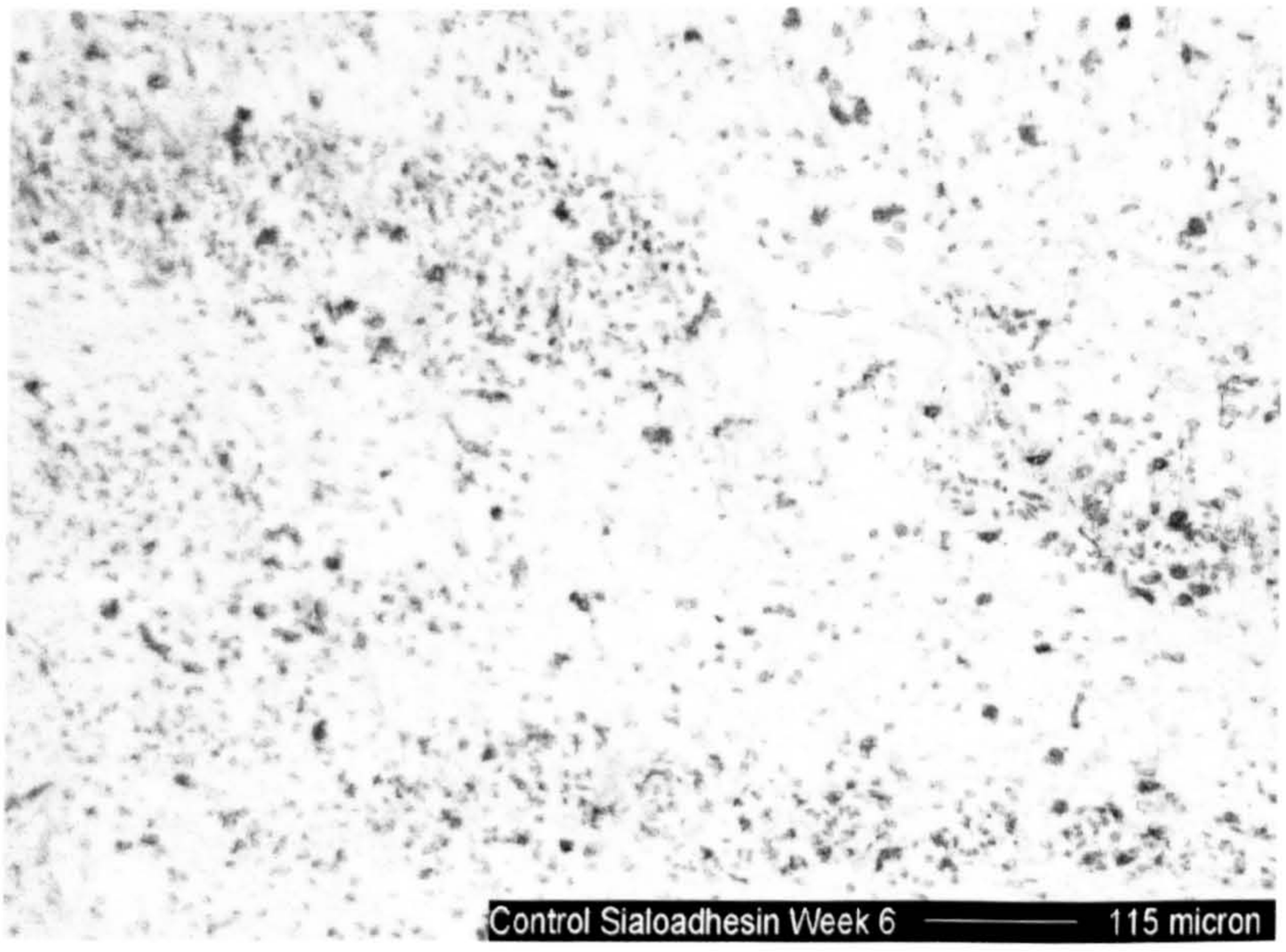


Week 6

Group 4 (Control) - There is little difference in the number of tissue macrophages at week 6 when compared to week 0



Week 0



Week 6

4.2.10 Cytokines

Cytokines, produced within cells leech out and were identified by antibodies for the specific cytokine within the wound bed tissue. Hence the intensity of staining of the sections with a specific antibody at week 6 was compared to the intensity at week 0 sections as previously described in chapter 3.2.11.2.2. A descriptive comparison was made between the two time frames for each patient.

4.2.10.1 Tumour Necrosis factor α (TNFα)

Staining was confined to cells in the vascular area of the sections, but was not always associated with the perivascualar regions of the sections. There is very little change in the mean score for the intensity of staining for TNFα at week 6 when compared to week 0 in the four treatment groups. (Table. 4-39) Comparing the groups for an increase or decrease in intensity of staining at the two time frames, exhibited a small increase in TNFα expression at week 6 when compared to week 0 in patients in Groups 2 and 3, and a small decrease in Group 4. (Table 4-40)

Table 4-39 The Mean Score for the Intensity of TNFα staining at Week 0 and Week 6

Groups	Mean Week 0 Score	Mean Week 6 Score
Gp1 (DGx12)	1	1
Gp2 (DGx4)	1	1
Gp3 (DGx1)	1	1.5
Gp4 (Control)	1	1

Table 4-40 Comparison of TNFα in staining intensity in patients at Week 6 to Week 0

Groups	Change in the Intensity of Staining at Week 6 when Compared to Week 0		
	Increase	**Decrease**	**Unchanged**
Gp1 (DGx12)	3	3	4
Gp2 (DGx4)	3	2	6
Gp3 (DGx1)	4	1	3
Gp4 (Control)	1	2	8

4.2.10.2 Interleukin 1β (IL1-β)

Staining seems to be concentrated in the cells in the perivascular area, and sparse staining was seen in areas where there was little vasculature.

There seems to be a small reduction in the IL1-β expression in patients in Group 2 at week 6 when compared to week 0, and small increase in Groups 1 and 4, but these changes are too small to be correlated to healing. (Tables 4-41 and 4-42)

Table 4-41 Mean Score for the Intensity of IL1-β Staining at Week 0 and Week 6

Groups	Mean Week 0 Score	Mean Week 6 Score
Gp1 (DGx12)	2	2
Gp2 (DGx4)	3	2
Gp3 (DGx1)	2	2
Gp4 (Control)	3	3

Table 4-42 Comparison of IL1-β in staining intensity in patients at Week 6 to Week 0

Groups	Change in the Intensity of Staining at Week 6 when Compared to Week 0		
	Increase	**Decrease**	**Unchanged**
Gp1 (DGx12)	4	2	4
Gp2 (DGx4)	2	5	4
Gp3 (DGx1)	2	2	4
Gp4 (Control)	4	3	4

4.2.10.3 Vascular Endothelial Growth Factor (VEGF)

Staining for VEGF is distributed in the peri-vascular tissue with negative staining of the vascular endothelial lining.

There is very little change in the mean intensity of VEGF staining between the two sections at week 6 and week 0. Patients in groups 2 and 3 however, demonstrated a reduction in the VEGF staining at week 6 when compared to week 0. (Tables 4-43 and 4-44)

Table 4-43 Mean Score for the Intensity of VEGF Staining at Week 0 and Week 6

Groups	Mean Week 0 Score	Mean Week 6 Score
Gp1 (DGx12)	1	1
Gp2 (DGx4)	1	0
Gp3 (DGx1)	1	0.5
Gp4 (Control)	1	1

Table 4-44 Comparison of VEGF in staining intensity in patients at Week 6 to Week 0

Groups	Change in the Intensity of Staining at Week 6 when Compared to Week 0		
	Increase	Decrease	Unchanged
Gp1 (DGx12)	3	3	4
Gp2 (DGx4)	0	5	6
Gp3 (DGx1)	1	2	5
Gp4 (Control)	3	3	5

4.2.10.4 Basic Fibroblast Growth Factor (bFGF)

bFGF usually stains an area adjacent to the blood vessels, and sometimes it can be seen to highlight the endothelial lining. There is very little change in the density of bFGF staining between the two sections at week 6 and week 0. There is a decrease in bFGF density at week 6 when compared to week 0 in patients in Group 2 and an

increase at week 6 when compared to week 0 in patients Groups 3 and 4. (Tables 4-45 and 4-46)

Table 4-45 Mean Score for the Intensity of bFGF Staining at Week 0 and Week 6

Groups	Mean Week 0 Score	Mean Week 6 Score
Gp1 (DGx12)	3	3
Gp2 (DGx4)	2	2
Gp3 (DGx1)	2	3
Gp4 (Control)	2	2

Table 4-46 Comparison of bFGF in staining intensity in patients at Week 6 to Week 0

Groups	Change in the Intensity of Staining at Week 6 when Compared to Week 0		
	Increase	Decrease	Unchanged
Gp1 (DGx12)	2	2	6
Gp2 (DGx4)	3	4	4
Gp3 (DGx1)	4	1	3
Gp4 (Control)	4	3	4

4.2.10.5 Transforming Growth Factor β (TGF-β)

Staining to TGFβ is usually found within the ground substance that makes up the granulation tissue, and blood vessels are specifically negatively stained.

There was very little change in the mean intensity density of TGFβ staining at Week 0 and Week 6. However, in all four groups, there was an increase in the intensity of staining at week 6 when compared to week 0. (Tables 4-47 and 4-47)

Table 4-47 Mean Score for the Intensity of TGFβ Staining at Week 0 and Week 6

Groups	Mean Week 0 Score	Mean Week 6 Score
Gp1 (DGx12)	1	1
Gp2 (DGx4)	1	1
Gp3 (DGx1)	1	1.5
Gp4 (Control)	1	1

Table 4-48 Comparison of TGFβ in staining intensity in patients at Week6 to Week 0

Groups	Change in the Intensity of Staining at Week 6 when Compared to Week 0		
	Increase	Decrease	Unchanged
Gp1 (DGx12)	3	0	7
Gp2 (DGx4)	3	1	6
Gp3 (DGx1)	2	0	6
Gp4 (Control)	4	3	4

5 CHAPTER FIVE: DISCUSSION

Professionals and health care workers have recognised the difficulties that can be associated with achieving wound closure, with the projected demographic changes within the UK, resulting in an increase in the elderly population in the coming years. Additional resources for wound care will be required to meet the increase in problems within this group, and hence a considerable amount of attention has been placed on wound healing in the last ten years

It has been estimated that chronic wounds such as pressure sores, diabetic ulcers, venous and arterial ulcers, contribute to morbidity and occasionally mortality, in patients. This incurs a considerable cost to the National Health Service. It has been estimated that the financial burden of venous disease in Britain is between £294 – 650 million per year, depending on the variation in practice, and the location of care provision, (eg. Community or out-patient clinic) (Price P et al., 2000).

Venous leg ulcers affect about 1% of the adult population at some time during their life-time. In the over 65s this can increase to around 3% of the population group, from 3/1000 at age 61 – 70 years, to 20/1000 in individuals aged 80 years and over. (Callam MJ et al., 1985, Baker SR et al., 1991, Callam MJ, 1992).

In the United States, treatment costs are $3 billion dollars with a loss of 2 million work days per year (McGuckin M et al., 2002). Some ulcers can be difficult to heal. A 5 year, prospective, cohort study of 382 patients reported that in patients surviving 5 years, 38% still had open ulcers and 4% required amputation (Nelzen O et al., 1997).

The most common types of leg ulcers seen in UK clinics are venous (70-80%) (Callam MJ et al., 1987) or arterial in origin (20-30%) (Callam MJ et al., 1987)

Disruption of the ordered progression towards healing, in patients can result in a chronic wound as seen in venous leg ulcers. Although venous leg ulcers do not

usually cause the loss of a limb, they can cause major morbidity and sometimes life threatening cellulitis.

Barwell et al in 2000, has shown that following surgical correction of isolated superficial venous reflux in ulcerated legs, 12 - and 24 - week healing rates for the operated leg were 50% and 72%, when compared to 62% and 74% for the non-operated legs. However, in this study ulcer recurrences were 14%, 20%, and 26% at 1, 2, and 3 years for the operated legs and 28%, 30% and 44% for non-operated legs, suggesting a long term effect on ulcer prevention if these groups of patients undergo surgery (Barwell JR et al., 2000). However, there exists a large population of patients, in whom surgery may not be possible due the pre-existence of other co-morbid medical conditions or damage to the deep venous system of the leg.

At present the choice of treatment of impaired healing or chronic wounds in clinical practice is largely dependent on the skill and experience of the medical/nursing practitioners. Traditionally, treatment has consisted of elevation, wound care, compression and patient education based on prevention. Compression therapy has been shown to be the most effective intervention for venous leg ulcers to overcome the effects of venous hypertension (Krishnamoorthy L and Melhuish JM, 2000). Even with optimal care (elevation of limb, compression and debridement) and follow up, venous leg ulcers recur in 29 – 59% of patients and are associated with significant disability and costs (Kowallek DL and De Palma RG, 1997, Morrell CJ et al., 1998).

Several risk factors including a large surface area, longer ulcer duration, the presence of fibrin over the wound surface (>50%) have been shown to play a role in the non-healing nature of venous leg ulcers despite compression therapy (Margolis DJ et al., 1999, Phillips TJ et al., 2000, Margolis DJ et al., 2000).

The future diagnosis and treatment of wounds, as well as the development of bioactive dressings, will bring a requirement for wound assessment to determine the most appropriate treatment. However, the mechanisms for the evolution of a normally healing acute wound into a non-healing chronic wound need to be defined.

Normal wound healing is a complex sequence of overlapping cellular and molecular processes, including inflammation, cell migration, angiogenesis, provisional matrix deposition, collagen deposition and re-epithelisation (Clark RAF, 1996).

The inflammatory phase of wound healing is the first and essential stage in the healing process, and it is assumed that this response has major influences on subsequent healing. After the first twenty four hours of injury, these cells are mainly comprised of macrophages and lymphocytes. The role of lymphocytes and their relative changes as healing proceeds has been described by several authors in the past (Martin CW and Muir IF, 1990, Fishel RS et al., 1987, Peterson JM et al., 1987).

This work describes in detail the various aspects of the role of macrophages and their various stages of differentiation and activation stages in acute and chronic human wounds.

5.1 PILOT STUDY

Macrophages are known to direct new tissue formation, by producing an array of bioactive substances, including growth and regulatory factors. (Hunt TK et al., 1984)

It was hypothesized that there would be major differences in the level of activation of wound macrophages between acute and chronic wounds. Ten acute and ten chronic wounds were assessed to ascertain the possible observable differences in the macrophage subpopulation in the two wound types.

5.1.1 Biopsies

To standardise collection the biopsies were obtained from the centre of the wound bed. The data was standardised by analysing the positively stained cells in the vascularised areas of the sections.

5.1.2 Specimen preparation

For the majority of histochemical methods the technique of cutting sections on a cryostat and allowing them to thaw to room temperature, maintains good morphological relationships, and produces minimal changes to biological structures.

This method was first described by Simpson in 1941, (Simpson WL, 1941), and was based on the quenching of the tissue in Isopentane in liquid nitrogen to inhibit autolysis, prevent putrefaction, diffusion, dissolution of substances within the tissues, and the formation of large ice crystals.

5.1.3 Immunohistochemical processing of the slides

The purpose of fixation is to preserve all the components of the tissue in their true situation without diffusion and also minimize tissue damage from osmosis. For immunohistochemistry, the antigen to be localized must be made insoluble but available for reaction with the applied antibody.

Acetone is a protein precipitant fixative. It precipitates proteins by destroying the hydrophobic bonds that hold the tertiary (three dimensional) structure of the protein molecules. The primary and secondary structures of the protein are left intact, so the amino acid sequences acting as antigens remain available for antigen-antibody reaction to occur. Hence it is suitable when the antigen is a large protein. Acetone fixation also allows a wide range of of primary antibodies to be used without destroying the epitopes they are trying to identify.(Bankcroft JD and Cook HC. 1994)

Formaldehyde fixatives are cross linkers, i.e they form links between reactive end-groups of adjacent protein chains. Such proteins can only retain their antigenicity if the cross-linking does not affect the amino acid sequences that bind to the antibody. Cross linking fixatives are often essential for fixing small proteins which are so soluble that they tend to diffuse and disperse and hence not long enough to be made insoluble by precipitant fixatives. (Pearse AGE 1980, Hopwood D. 1985, Puchtler H and Meloan SN. 1985, Fox CH et al. 1985)

Both fixatives have been tested for this study and it was found that acetone fixation yielded the best results, and hence the formaldyhyde results were not discussed in this thesis.

The techniques of immunohistochemistry currently used today owe much to the extensive work done by key researchers over the last sixty years. One of the most

significant influence in its development was a paper by Coons et al, in 1941 describing the fluorescent-labeled antibody technique. (Coons AH et al. 1941). Whilst useful in some diagnostic areas, such as determining the nature of protein deposits in renal and skin diseases, it had its limitations. One of its main drawback was its difficulty to demonstrate morphological details of cells.

Enzyme labeling of antibodies followed by the appropriate substrate/chromogen mixture such as DAB reported by Nakane and Pierce in 1966, overcame this disadvantage as the labeled cells could be counterstained with traditional nuclear stains such haemotoxylin. (Nakane PK and Pierce GB 1966)

The Streptavidin/biotin technique used in this study is currently the most popular system. This consists of a tetravalent glycoprotein from egg white which has a high affinity for biotin. The biotin can then be linked to specific antibodies without causing any loss in their specific binding activity. This amplifies the presence of the antigen without increasing the background staining (Hsu SM, 1990 Hsu SM et al., 1981)

5.1.4 Analysis

Previously work from this unit has demonstrated that there are low numbers of macrophages in undamaged normal skin specimens when compared to acute wounds (with a mean count of 10.8 positive cells per x 40 hpf, when compared to a mean count of 44.6 positive cells per x 40 hpf in acute wounds) (Boyce DE, 2000).

This study, demonstrates that macrophages are present in large numbers in both acute and chronic wounds, as demonstrated by the large number of cells stained by the pan-macrophage marker CD68, but the subpopulation of macrophages can vary. There are significantly (Mann Whitney U test) large numbers of early stage macrophages

(27e10^{+ve} cells) within the acute wounds when compared to chronic wounds ($p \leq 0.01$) and significantly larger numbers of tissue macrophages (SR^{+ve} cells) within the chronic wounds when compared to the acute wounds ($p \leq 0.01$). The number of primed macrophages (MR^{+ve} cells) is present in large numbers in both wound types. This suggests that both wound types demonstrate an array of macrophage subpopulations. In the acute wounds, one might postulate that there seems to be a continuous influx of monocytes. These differentiate into early stage macrophages expressing the 27e10 antigen, which become primed expressing the MR antigen. These primed cells then become activated secreting growth factors or becoming directly involved in the healing process, and removed from the wound environment, allowing more room for further monocyte infiltration from the blood stream. This could explain the presence of a small number of tissue macrophages observed within acute wounds.

In the chronic wounds however, the primed macrophages fail to undergo activation and hence mature into SR^{+ve} tissue macrophages. Cassidy et al in 1989 and Perry et al in 1992 have already suggested possible roles for this subset of macrophages.They function by adhering to other cells in the wounded tissue and help to minimise the tissue damage by activated granulocytes. They aid in the clearance of senescent neutrophils within chronic wounds, and through their direct receptor-cell interaction, influence cells such as lymphocytes and thymocytes, influencing the behaviour of these cells in chronic inflammation. These cells may also act in an unknown manner that is still unclear at present by preventing or reducing further influx of monocytes into the wound area. This is reflected in this study by the presence of small numbers of early stage macrophages in chronic wounds. (Cassidy LF et al in 1989, Perry VH et al., 1992).

5.2 DERMAGRAFT STUDY

The Unna boot produced in the 19th century was the first recorded bandage specifically designed to apply compression to the lower leg. The bandage is impregnated with zinc oxide which reduces the bacterial load within the leg ulcers and increases the inflammatory response in granulation tissue, and applied in a spiral pattern with a 50% overlap (Agren MS et al., 1993). Compression therapy in one form or the other has been the main stay of patient treatment for venous leg ulcers and has proved to be the most effective non-invasive treatment to-date. (Falanga V, 1993, Partch H, 1995, Blair SD et al., 1998)

When conservative therapy with compression fails, attempts at wound closure have included the correction of the underlying venous insufficiencies and autologous split-thickness skin grafting, but these involve increased costs and hospitalisation (Lofgren KA et al., 1965, Cikrit DF et al., 1988). As these patients tend to be more elderly they may suffer from other co-morbid conditions which may play a role in the chronicity of the ulcers and limit the type of invasive surgery that can be performed.

Major technical advances in tissue engineering over recent years have made it possible to culture human keratinocytes in laboratory conditions. Such autologous sheets of keratinocytes have been used in the treatment of venous leg ulcers with encouraging results (Leigh IM et al., 1987, Phillips TJ et al., 1992).

There are several limitations to the use of cultured keratinocytes, (or cultured epidermal autografts - CEA).

1. It can take up to three weeks to obtain sufficient amounts of CEA sheets for grafting

2. The average 'take' of these grafts is less than split skin grafts (Phillips TJ and Gilchrist BA. 1992)
3. The graft 'take' is worst in areas of high pressure or shear forces – which is believed to be due to the inadequate formation of epidermal keratinocyte anchoring fibrils and the absence of rete ridges
4. Marked wound contracture, and
5. The high cost of graft production.

There have been more promising results with the incorporation of dermal components to CEA cultures. This seems to improve the take and reduce contraction of the grafts (Couno CB et al., 1987, Odessey R, 1992). There are some commercially available dermal substitutes, - Apligraf™, Alloderm™, and Integra™, which provide the dermal component to increase adherence of CEA (Falanga V et al., 1998, Wainwright D et al., 1996, Heimbach D et al., 1988, Freedlander E, 1998, Pomahac B et al., 1998, Hansborough JF and Franco ES, 1998).

Dermagraft™(DG), a tissue engineered human replacement has previously been shown to improve healing in diabetic patients with plantar foot ulcers. It is believed that DG stimulates healing in this group of patients by providing metabolically active products such as matrix proteins and growth factors directly to the wound bed (Gentzkow GD et al., 1996.).

It has been postulated in chapter 2 of this thesis that DG might exhibit the same stimulatory effect in achieving healing in other recalcitrant wounds such as venous leg ulcers.

There was a need for a controlled trial to assess the efficiency of DG on venous leg ulcer patients and to monitor the selected biological markers in this thesis. This was

assessed by applying the DG pieces to the wound bed and the conventional therapy of compression in the form of a four-layer compression bandage in the promotion of healing venous leg ulcers. This was compared with four-layer compression alone. It was also important to try and determine the optimal treatment dose of DG in achieving wound closure. Studies in patients with diabetic foot ulcers have demonstrated that weekly application of DG for at least eight weeks is needed to achieve healing (Naughton G et al., 1997, Pollack RA et al., 1997),

5.2.1 Patient population

The sample size in each treatment arm of the study was small; this being further reduced by patient withdrawal, insufficient biopsy size, and the availability of paired biopsy samples.

Although in total, 5 out of the 6 centres contributed 27 patients for this study, Cardiff contributed 26 patients (almost 50%) of the study population. This may be because Cardiff is one of the few tertiary multidisplinary specialist referral centres in the UK and have a greater patient catchment when compared to the other centres recruiting patients in a hospital setting.

However, it was noted that the median ulcer size and median duration of the study ulcer were of similar magnitude for all study groups indicating a patient population of those with 'harder to heal' venous ulcers.

The results demonstrate that the largest number of patients that achieved complete wound closure were treated with 12 or 4 pieces of DG (5/13 and 5/13 respectively). This is compared to only 2/13 in the control group that was treated with compression only. This is similarly reflected in a greater reduction in the size of the ulcer at week

12 for the 12 and 4 pieces of DG groups (81.4 and 88.6% respectively). The control group demonstrated a reduction in the ulcer size in only 78.1%.

Considering that DG is an expensive product, the 4 pieces of DG regime (group 2) would be the best suggested treatment that achieves the most economic response both in terms of healing and cost.

It is suggested that for the future pivotal study for DG treatment of venous leg ulcers, 4 pieces of DG would be the prescribed treatment regime.

5.2.2 Safety

The most commonly reported adverse event for the patients was wound infection. Wound infection was assessed by the criteria described by Cutting and Harding in 1994 (Cutting K and Harding KG, 1994). This accounted for 49% of all noted adverse events. However there were no significant differences in these adverse events between the treatment groups or the control group.

Diagnosis of wound infection was a clinical observation made by the investigators at the various centres and any treatment .instigated was in accordance with the local practice of these centres. Wounds were not routinely swabbed as numerous authors have published data suggesting that all wounds if swabbed would demonstrate the presence of bacteria. Wound colonisation is not always the same as invasive infection, thus raising the reliability of wound swabs in these situations (Chesham J and Platt D, 1987, Trengrove NJ et al., 1996, Thomson PD and Smith DJ Jr, 1994.). Although efforts have focused on identification and quantification of organisms involved, the predictive value of this variable is limited. It has also been found that large acute wounds react differently to skin flora organisms than small chronic wounds. It is

thought that the most useful method for determining the microbial load and the presence of invasive pathogens in a wound is to biopsy the wound when there is a clinical suspicion of infection. (Fowler E. 1998, Neil JA and Munro CL. (1997). However the validity and the value of single biopsy specimen in chronic wounds is debatable (Schnieder M et al.1983), traumatic to the patient and facilities for the analysis of such tissue are not widely available.

A more accurate predictor of colonisation or infection is the behaviour of the wound and hence careful and frequent assessment of the patient and the wound, as practised in this study, can forecast when the offending organisms, their toxins or both reach sufficient concentrations to overwhelm the individual hosts' defences. Thus it was decided in this study to rely on the experiences of the clinical investigators rather than a laboratory test to diagnose wound infection.

Submitting the patients to more biopsies to possibly qualify wound infection was not thought to be suitably ethical.

DG being a bioengineered product may stimulate an inflammatory response which might be incorrectly interpreted as wound infection. However the application of DG onto the wound bed did not adversely affect the infection rate in the treated groups compared to the control group.

5.2.3 Histological

Sampling times can affect the results of any study. Our histological sampling times had been chosen at weeks 0 and 6. This allowed for a reliable histological base sample and the best time frame in which to observe the effects of DG as demonstrated by previous work presented from this unit.

5.2.3.1 Wax sections

Extracellular matrix (ECM) and collagen distribution

Chronic wounds such as venous leg ulcers usually contain large amount of fibrin intercalated among the collagen bundles, and are particularly prominent around the vessels forming a fibrin cuff. It has been proposed that the perivascular fibrin cuffs inhibit the diffusion of oxygen form the blood vessels to the tissues, and thus impede wound repair (Burnand KG and Browse NL, 1982). Fibrin cuffs have been shown to become highly cross linked to the collagen bundles. (Mosesson MW et al., 1989, Shainoff JR et al., 1991, Siebenlist KR and Mosesson MW, 1992) The matrix exhibits reduced porosity preventing cell penetration, reduced susceptiblity to fibrinolysis, and alter the interaction between the cells and the cytokines (Brommer EJP and Van Bocke LJH, 1992). It has also been proposed that the fibrin cuffs may trap growth factors and hinder their distribution into the wound (Falanga V and Eaglestein WH, 1993), thus the wound environment essentially becomes nutritionally depleted without function.

By introducing Dermagraft with its fresh stock of fibroblasts producing proteolytic enzymes and growth factors, may first trigger proteolysis of the ECM and stimulate the wound to heal. Histologically, this may result in the patchy appearance of the ECM, as seen in the biopsies of those patients who eventually had a reduction in the size of their ulcer or complete closure, in this study.

A correlation was observed between the depth of collagen deposit at the base of the ulcer and healing. The presence of a 'dense scar' (described as orientated fibres

composed of densely packed fine collagen) correlated with a reduction in the ulcer size and wound closure. In surgical wounds, such as pilonidal excisions which were allowed to heal by secondary intention, it has been shown that as healing progresses, the depth of the collagenous layer increased significantly over time and accounted in part to the reduction in the wound volume (Berry DP, 1997). Similar increases in the collagen layer at the base of venous ulcers (described as orientated fibres composed of densely packed fine collagen), seen in this study, may contribute to the reduction in the volume of the ulcers.

Blood vessels

In acute tissue injury destruction and disruption of the cells causes proteolytic enzymes to be released into the connective tissue. This degrades the ECM and causes fragmentation of the basement membrane (Kalebic T et al., 1983). This allows endothelial cells to migrate into the wound in response to fibroblast growth factor (FGF), fibronectin fragments, heparin, and other endothelial cell chemoattractants. As the endothelial cells migrate into this environment, they form tubes and facilitate adhesion and migration of the neovascularature. The FGF and other mitogens stimulate endothelial proliferation (Folkman J and Klagsbrun M, 1987), resulting in a continual supply of endothelial cells for capillary extension. Capillary sprouts eventually branch at their tips and join others to form capillary loops and capillary plexuses. Histologically, these plexuses will be randomly distributed within the tissue. It has been demonstrated in this study that a random distribution of blood vessels in the biopsy specimens was related to a decrease in the wound volume. However due to

the small number of patients in each group, this observation was not statistically significant in any of the four groups or to the dosage regime of DG.

Patients who received 4 pieces of DG also exhibited a higher number of blood vessels at week 6 compared to week 0, than the other three groups. This was related to wound closure or in the reduction of the ulcer size ($p<0.05$)

As stated before, prominent fibrin cuffing around the vessels impairs wound healing. In this study although the degree of fibrin cuffing did not change markedly between the two time periods, a low proportion of fibrin cuffing either at week 0 or week 6 was associated with a reduction in ulcer size.

Bergan et al. in 2001, demonstrated that if the amount of fibrin cuff around blood vessels could be inhibited, in their case with the application of Daflon(R), the amount of fibrin cuffing is reduced and this is seen clinically by acclerated healing of venous ulcers. (Bergan JJ et al., 2001) This study supports the findings observed in this thesis.

5.2.3.2 Frozen Sections

Cell Pattern and Distribution

Neutrophils

An early event in healing wounds is the migration of neutrophils into the wound environment, closely followed by monocytes, but as there are more neutrophils in the circulation, they appear in the wounded tissue in greater numbers. A variety of chemotactic agents attract both cell types to the site of injury (Williams TJ, 1988). Neutrophils destroy any foreign contamination or bacteria via phagocytosis and

subsequent enzymatic and oxygen free radical mechanisms (Tonnesen MG et al., 1988, Elsbach P and Weiss J, 1992, Klebanoff SJ, 1992). Neutrophil infiltration usually ceases within a few days, and most of the neutrophils become trapped within the eschar, which sloughs during tissue regeneration. As healing progresses there is a resolution of the mononuclear inflammatory response. Persistent wound contamination with debris, foreign bodies or bacteria results in a persistent neutrophil inflammatory response and their accumulation in chronic wounds. They may continue to produce enzymes such as elastase that breakdown connective tissue and impair wound healing. As healing proceeds, there is a reduction in the mean number of neutrophils in acute wounds (Hofer HP et al., 1994). In this study, there was a gradual reduction in the mean number of neutrophils within the chronic wound between week 0 and week 6 biopsies, but it was not related to a reduction in the ulcer size or wound closure. The maximum reduction in the mean number of neutrophils was seen in the control group. DG on the wound surface in the treatment groups may continue to stimulate the presence of neutrophils in the tissue environment as it is a foreign material. Thus is reflected in the slower reduction of neutrophil counts in the treatment group.

5.2.4 Inflammatory Cells

5.2.4.1 Lymphocytes

Lymphocytes comprise a significant proportion of the mononuclear cell infiltrate, but little is known of the role of these cells in the healing process. They are a heterogenous group with a wide variety of functions. Natural Killer (NK) cells are a

small part of the lymphoid pool and are concerned with viral and tumour immunity. B lymphocytes are responsible for the 'antibody-mediated' immunity, and T lymphocytes for 'cell-mediated' immunity.

B lymphocytes

Martin and Muir in 1990, suggested that these cells are not routinely present in human wounds, but their data was limited to biopsies obtained from wounds at day 1 and day 3 (Martin CW and Muir IF, 1990.).

In this study, small mean counts of $CD19^{+ve}$ B lymphocytes were present in chronic wounds both before and after treatment, and there was a non significant increase in the mean count of $CD19^{+ve}$ B lymphocytes at Week 6. The significance of the presence of B lymphocytes within wounded tissue is still unclear.

It could be postulated that their presence is related to ongoing wound contamination of some type. This could be due to the progressive colonisation of bacteria in wounded tissue. It is known that some bacterial polysaccharides, polymeric proteins and lipopolysaccharides, called 'thymus independent (TI)' antigens, may exhibit an intrinsic activity which can stimulate proliferation and differentiation of B lymphocytes in the absence of T lymphocyte helper cells. (Janeway C and Travers P, 1996)

If infection and chronicity were the main reasons for their presence, then one would expect to find a large mean count of B lymphocytes within the chronic wounds. The small mean count of $CD19^{+ve}$ B lymphocytes at week 0 and its increase at week 6 were more obvious in the treatment groups and hence one could postulate that their continued presence be due to some unknown effect of DG on the wound bed.

In 1998, Cowin et al, demonstrated the presence of B lymphocytes in adult murine wound tissue but not in foetal wounds (Cowin AJ et al., 1998). They postulated that B lymphocytes contributed to the fibrotic healing seen in adults, but which was absent in foetal healing. Thus the rise in the numbers of B lymphocytes at week 6 when compared to week 0 in our patients may reflect similar roles for these cells in the wound healing process. It is known human adult cutaneous wound healing results in the formation of scar tissue, and the B lymphocytes may aid the formation of the fibrotic scar tissue by a yet unrecognised mode of action. This view however, is in conflict with the 'standard' opinion, which assumes that B lymphocytes have no role in wound healing (Schaffer M and Barbul A, 1998, Martin CW and Muir IF, 1990)

T lymphocytes

The potential role for T-lymphocytes in wound healing have largely been limited to *in vivo* studies on murine models (Peterson JM et al., 1987, Fishel RS et al., 1987, Barbul A, 1988). Total depletion of T lymphocytes demonstrates a reduction in collagen and thus the mechanical strength of wounds. The selective depletion of $CD8^+$ lymphocytes result in enhanced wound healing. Depletion of $CD4^+$ lymphocytes demonstrated no significant delay in wound healing. The authors concluded that $CD8^+$ lymphocytes were responsible for a 'down-regulation' of the healing process. Other authors have shown that $CD4^+$ lymphocytes or their cytokines are responsible for the 'up-regulation' of wound healing in rat epitenon models (Wojciak B and Crossan JF, 1994).

It has been demonstrated in this study that there is an increase in the mean count of $CD3^+$ (pan T lymphocyte marker) at week 6 when compared to week 0 in those

groups treated with four or more pieces of DG and the control group. The increase in the treated groups may be related to repeated dosage of DG when compared to a single dosage at week 0, but the increase in the control group seems more difficult to explain. The significance of this finding remains unclear.

The relatively unequivocal changes in the $CD4^+$ and $CD8^+$ cells in response to treatment may be a reflection of the chronicity of the wound. This is reflected by the low CD4:CD8 ratios in this study. Boyce et al, demonstrated that the mean CD4:CD8 ratio in chronic wounds were low when compared with acute healing wounds which initially have a high ratio reducing to lower levels as healing proceeds. The mean CD4:CD8 ratio in undamaged skin seems to be of similar magnitude to chronic wounds. (Boyce DE et al., 2000)

The similarities in the ratios between undamaged skin and chronic wound as demonstrated in this study, suggests that DG does not regulate T lymphocytes in chronic wounds but mediate wound closure by other mechanisms, possibly by providing the necessary cytokines.

5.2.4.2 Macrophages

Macrophages are known to be essential for normal healing. Their initial role is in the inflammatory and debridement phase before fibroplasia (Jiang-Ping C et al., 1991, Nielsen BW et al., 1994). They then become the predominant cell prior to fibroblast proliferation and it has been demonstrated that they play a major role in the orchestration of wound repair (Riches DWH, 1996).

Large numbers of macrophages have been shown to be present in acute and chronic wounds when compared to undamaged skin. (Boyce DE, 2000) The pilot study demonstrated that there is an accumulation of early stage macrophages within acute wounds when compared to chronic wounds, and a significant accumulation of tissue macrophages within the chronic wounds when compared to acute wounds.

In this study, the different macrophage sub-populations within the four groups prior to initiation of treatment was of similar magnitude.

At Week 6, it can be seen that in general the mean count of early stage macrophages increased in all four groups with the greatest increase observed amongst those patients treated with four pieces of DG. When comparing the changes in the mean count of tissue macrophages at Week 6, there seems to be a general reduction in the counts with a maximal reduction seen in those patients treated with four pieces of DG.

The increase in the early stage macrophages and a decrease in the tissue macrophages in all four groups may also be due to the compression therapy all the patients received. This study however, demonstrates an observable increase in the early stage macrophages and a decrease in the tissue macrophages in the treated group (maximal in Group 2), suggesting that DG must contribute to the 'switching' of chronic wounds to acute status, encouraging healing to take place. It can also be inferred from the

results that application of DG at regular intervals provides the necessary local favourable environment for healing to take place.

5.2.4.3 Cytokines

Cytokines are regulatory proteins with an array of names which has arisen because of co- discovery by several different investigators in different inflammatory, and non-inflammatory cells at similar times. The term cytokine is the most general definition and not restricted to the immunohaemopoietic system (Cohen S et al., 1974).

Macrophages play multiple roles in both the degradative and reparative phases of wound healing. They regulate the various phases of wound healing by producing an array of cytokines which have the potential to improve wound healing by several mechanisms

(1) Attraction of inflammatory cells and fibroblasts into the wound via the secretion of chemotactic agents

(2) Stimulate cellular proliferation by acting as mitogens

(3) Stimulate angiogenesis and regulate the in growth of new blood vessels

(4) Have an important effect on the production and degradation of extracellular matrix, and

(5) Influence the synthesis of other or identical cytokines and growth factors by neighbouring cells. (Greenhalgh DG, 1996)

The more important cytokines involved in wound healing such as those targeting mesenchymal cells, regulating neovascularisation, remodeling were assessed between wound bed sections at week 0 and week 6.

Tumour Necrosis Factor (TNFα)

TNFα, a pro-inflammatory cytokine is released primarily by the macrophage lineage. It is crucial in initiating the immune cascade during the host response to injury or bacteria. TNFα is involved in the recruitment and maturation of the cellular component of inflammation, which includes the up-regulation of cell-surface adhesion molecules that plays a vital role in the immune cell to endothelium interaction, thus facilitating neutrophil chemotaxis (Omann GM and Hinshaw DB, 1997, Moser R et al., 1989).

Its main effects include haemostasis, increased vascular permeability and proliferation. It also promotes many cellular metabolic events that increase the supply of nutrient substrates and acute-phase protein synthesis essential for wound healing (Fong Y and Lowry SF, 1996).

Feiken et al, in 1995, observed that TNFα levels are usually detected in acute experimental wounds in the mouse model after 12 hours post wounding, peaking at 72 hours, and then tailing off quite rapidly (Feiken E et al., 1995)

Excessive production of TNFα has been demonstrated to lead to adverse clinical outcomes such as cahexia in cancer patients and some of the severe effects seen in gram negative sepsis (Larrick JW and Kunchel SL, 1988). It is partly mediated through the recruitment and activation of neutrophils, (Bevilacqua MF et al., 1986, Nawroth PP and Stern DM, 1986). In this situation, the cells are responsible for increased tissue damage through the production of proteolytic enzymes and arachidonic acid metabolites (Striter RM et al., 1990, Tracey KJ et al., 1988).

Rapala et al demonstrated that TNFα reduced granulation tissue ingrowth after 7 days in acute experimental wounds in a dose-dependent manner, but the effects were not

long lasting. Similarly, Buck et al. observed that chronically elevated levels of TNFα in rats impaired wound healing, but these effects were partially reversed by the addition of a TNFα anatgonist in another study (Rapala K et al., 1991, Buck M et al., 1996)

Wallace and Stacey demonstrated no cause effect relationship between elevated levels of TNFα in non-healing verses healing venous leg ulcers in humans (Wallace HJ and Stacey MC, 1998).

In this study low concentrations of TNFα (as demonstrated by the weak staining of the antibody) were seen within wound beds at week 0 and week 6, in all four groups, irrespective of any wound size reduction or treatment. Murphy et al has illustrated that compression therapy can result in wound closure, and reduce the TNFα levels within the wound. Hence the low TNFα levels observed in this study at week 0 and week 6 may be due to compression therapy which preceded DG application by two weeks and DG itself may have no effect on the level of TNFα in the wound environment (Murphy MA et al., 2002)

Interleukin 1-beta (IL1β)

IL1-β is primarily produced by cells of the macrophage lineage, and by keratinocytes in active wounds (Sauder DN et al., 1990, Goretsky MJ et al., 1996). It is also produced by endothelial cells, fibroblasts and smooth muscle cells. It activates neutrophils, upregulates adhesion molecules, promotes chemotaxis and supports endothelial cells to secrete pro-inflammatory cytokines (Fong Y and Lowry SF, 1996, Loppnow H et al., 1998).

IL1-β become detectable within the first 24 hours after injury, and peaks between the first and third day, declining rapidly to undetectable levels within seven days (Goretsky MJ et al., 1996, Fahey TJ et al., 1990).

Several studies have demonstrated that elevated levels of IL1-β contribute to wound chronicity, thus the early beneficial effects of IL1-β in wound healing becomes disturbed if high levels persists after the first week (Trengove NJ et al., 2000, Barone EJ et al., 1998). Work from the author's laboratories, has demonstrated that high levels of IL1-β correlates with the non-healing status of venous leg ulcers (personal communication).

In this study, IL1-β staining was shown to be more intense than TNFα, and is concentrated within the cells in the perivascular area. In group 2, (those patients treated with four pieces of DG) there is a reduction in the intensity of staining for IL1-β at week 6 when compared to week 0. This group has a higher proportion of patients with healed wounds compared to the control group. Patients in group 1(those that received twelve pieces of DG) who also had a higher proportion of healed wounds, did not demonstrate a similar reduction in the intensity of staining for IL1β at week 6 when compared to week 0 however. As stated previously, fibroblasts produce IL1β. The positive staining for IL1β in this group of patients (Group 1) may actually originate from fibroblasts that are present in DG that is applied onto the wound bed weekly rather than endogenous production of IL1β within the stimulated wound bed.

Vascular Endothelial Growth Factor (VEGF)

VEGF is released by macrophages and fibroblasts in wounded tissue (Frank S et al., 1999). The levels rise reaching detectable levels in the acutely wounded tissue at 24

hours. The levels continue to rise steadily during the first week peaking at about day 5, then fall becoming almost undetectable by the end of the second week. The main trigger for the production and release of VEGF is thought to be wound hypoxia and nitric oxide production (Nissen NN et al., 1998).

The exogenous administration of VEGF has demonstrated an improvement in granulation tissue formation in normal and hypoxic experimental wounds (Corral CJ et al., 1999).

Other cytokines can affect the production and effects of VEGF. It has been recently demonstrated that high levels TNFα can down regulate the production of VEGF within a wound (Patterson C et al., 1996, Guo DQ et al., 2000).

A recent study by Inoki et al, demonstrated that the angiogenic effects of VEGF can be neutralised by adding connective tissue growth factor to recombinant VEGF. When this mixture is injected in to mice no neovascularisation is seen (Inoki I et al., 2002).

VEGF may contribute to the angiogenic stimulus in wounds either by direct effects on the proliferating and migrating endothelial cells or indirectly by affecting persistent permeability at the level of existing microvessels (Nissen NN et al., 1998). Hence, VEGF production and VEGF-mediated angiogenic activity would arise in the early hypoxic wound and then fall when neovascularisation is complete or wound perfusion is restored, but may also be reduced if high levels of TNFα were present.

In this study, VEGF staining was found to be distributed in the peri-vascular tissue with no staining of the vascular endothelial lining itself.

Group 2 demonstrates a reduction in the staining intensity at week 6 compared to week 0. This may be because adequate wound perfusion is restored resulting in a larger number of healed wounds, and the stimulus for continued VEGF production is switched off. In the small proportion of those wounds that remained unhealed in this

group, one might postulate that factors other than perfusion are preventing the wounds from achieving closure.

In group 1, DG may provide a continuous reservoir of VEGF and no detectable decrease in the intensity of staining is demonstrated within the week 6 samples.

The low grade in the intensity of staining of VEGF in all groups suggests that VEGF may not be needed in large concentrations to stimulate angiogenesis and other growth factors present in the wound environment may also have angiogenic effect.

Basic Fibroblast Growth Factor (bFGF)

FGF, is a family of proteins that play an important role as mediators of wound angiogenesis and epithelialisation. The most potent of these is bFGF (Adzich N, 1997). bFGF is released by macrophages and endothelial cells and stimulate fibroblast and keratinocytes proliferation and migration (Mustoe TA et al., 1991, Hebda PA et al., 1990). It promotes endothelial cell growth and migration that is essential for angiogenesis, prevents wound contraction and plays an essential role in collagen remodelling (Finetti G and Farina M, 1992, Ono I et al., 1999).

In acute wounds, bFGF levels peak in the post wounding period, falling to near non-detectable levels 24 hours later (Nissen NN et al., 1996).

It is believed that bFGF is stored in many cells in the uninjured tissue and is released into the wound when these cells become destroyed as a result of direct injury or by enzymes such as thrombin. This would explain the sudden release of bFGF in response to injury (Cordon-Cordo C et al., 1990, Muthukrishnan L et al., 1991, Ben-Ezra M et al., 1993). This is further supported by the absence of secretory signal peptides in the cells that store these proteins (Jaye M et al., 1986).

In this study, intense bFGF staining was visualised in the area adjacent to the blood vessels, and sometimes it can be seen to highlight the endothelial lining. There also seems to be a small increase in the staining intensity in the sections of the control group and Group 3, at week 6 when compared to the week 0. This could be due to the continued presence within the wound environment products such as bacterial degradation products or proteases which damages cells. In those groups that received more frequent dosing of DG, bFGF levels reduced slightly. This could be due the presence of neo-bFGF produced by the fibroblasts in DG.

Transforming Growth Factor -β (TGFβ)

In adults wounded cutaneous tissue heals by scarring. TGFβ is generally acknowledged to be the cytokine with the broadest range of activities in the repair of injured tissue, as a variety of cells produce and /or respond to it (Roberts AB and Sporn MB, 1990).

TGFβ is the general name for the family of naturally occurring polypeptides which have receptors on virtually every cell and exerts multiple regulatory effects on cell proliferation and differentiation (Cox DA, 1995).

It is released in the form of a large latent complex from the α-granules of platelets, when they degranulate in the presence of thrombin. It stimulates the migration of monocytes, lymphocytes, neutrophils, and fibroblasts in a dose-response manner (Wahl SM et al., 1987, Postlethwaite AE et al., 1987, Adams DA et al., 1991, Brandes ME et al., 1991).

In rats, injury releases stored TGFβ from degranulating platelets. Subsequently, injury induced expression of early genes contributes to further production and release of

TGFβ (Wang JY and Johnson LR, 1994). Thus levels of TGFβ remains high upto 14 days, peaking on days 7 to 9 post injury (Cromack DT et al., 1987).

Three isoforms exists in mammals, designated TGFβ-1, TGFβ-2, and TGFβ-3. It induces the production of procollagen type 1 and fibronectin, and inhibits metalloproteinases. The net effect is an increased deposition of collagen (Krishnamoorthy L et al., 2001).

Wysocki et al, in 1993, demonstrated that wound fluid obtained from chronic wounds to have abnormally raised levels of metalloproteinases and low levels of growth factors such as TGFβ which aids in the healing process, compared to acute mastectomy wound fluid (Wysocki AB et al., 1993).

In this study, however prominent TGFβ staining was found within the ground substance that made up the granulation tissue and blood vessels were specifically negatively stained. There was a small increase in the levels of TGFβ in response toDG in the treatment groups when compared to the control group. This supports our initial concept that once a wound attains certain amount of stimulation (in Group 3, one dose of DG), it can trigger a positive chain reaction that stimulates healing.

6 CHAPTER SIX: CONCLUSION

Knowledge of events which make up the inflammatory response in wound healing is still limited. The inflammatory phase of healing is the first and essential stage in the healing process, and it can be assumed that cells that participate in this response can influence subsequent healing. After neutrophils, the major cellular constituent of healing is the macrophage. The hypothesis underlying this thesis stems from animal studies which have shown that macrophages play a vital role in the orchestration and execution of both the degradative and the reparative phase of wound healing. It was thus assumed that one of the major differences between acute and chronic wounds may involve differences in the sub-population of wound macrophages and hence differences in the level of their activation within the wounded tissue.

In the first part of this thesis, (pilot study), it has been shown that more macrophages are present in wounded tissue than normal skin and different sub-population of macrophages are indeed present, with an accumulation of early macrophages within the acute wound and an accumulation of tissue macrophages within the chronic wounds.

In the next step of the thesis, The DG study was aimed to observe the changes in chronic wounds in response to the application of DG onto the wound bed in a 12 week study period, and whether it was safe to use in this sub-population of patients.

This was a feasibility study, with 63 eligible patients screened, and 53 patients being randomised into the four arms of the study.

It had been possible to show that treating the wound with DG 12 or 4 pieces resulted in more wounds healing completely by week 12, compared to compression therapy alone.

No major safety issues were identified concerning the use of DG. The most commonly reported adverse event was infection, but this phenomenon was not

uncommon in patients with venous leg ulcers. There were no notable differences in the safety profiles of the four treatment regimens.

Histologically, DG seemed to result in the patchy appearance of the ECM, as visualised in the biopsies of those patients who eventually had a reduction in the size of their ulcer or complete closure, but this appearance did not seem to be dose dependent. The reason for this observable trend remains unclear.

DG increased the level of collagen and this correlated with a reduction in the ulcer size and wound closure in some patients. In those patients treated with 4 or more pieces of DG showed a greater number of blood vessels at week 6 in the wound when compared to controls. It was also noted that a more random orientation of blood vessels correlated with increased ulcer healing, conversely, perpendicular alignment correlated with decreased wound healing, but DG had no visible effect on the orientation of the vessels.

Although there was a general reduction in the neutrophil count in all treatment groups at week 6 when compared to week 0, the most significant reduction in the mean neutrophil count was seen in the control group. Although the reason for this is unknown, one can postulate that DG itself, an allogenic material may have stimulated or produced chemoattractants for neutrophils.

There seemed to be no significant changes in the level of lymphocytes at the two time frames in the four treatment groups, but there was a significant accumulation of early stage macrophages and a significant reduction the mean count of tissue macrophages in those patients treated with 4 pieces of DG.

There also seemed to be very little change in the levels of cytokines within the wound bed at the two time frames. One may conclude that the ubiquitous presence of proteinases within the wound bed may have hydrolysed some of the cytokines

produced by the application of DG onto the wound, and a hence noticeable change was not apparent.

As each piece of DG was quite expensive, the above trends seemed to suggest that treatment with a four-piece regimen of DG may be sufficient to achieve wound closure and hence form the dose regimen for further pivotal studies. The pivotal study utilising a large patient population has now been commenced worldwide.

7 CHAPTER SEVEN: REFERENCES

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7.1 APPENDIX

Reagents

Tris Buffered Saline (TBS)

DAB enzyme substrate working solution from DAKO DAB tablet

Preparation of Vectastain working reagents

TRIS Buffered Saline (TBS)

Dissolve 80g sodium chloride and 6.05g of TRIS in 9 litres distilled water and adjust pH to 7.6, if necessary with 1M HCl. Make up volume to 10 litres with distilled water.

DAB enzyme substrate working solution from DAKO DAB tablets

1. *TRIS Buffer 0.005M pH7.6*

   Dissolve 1.52g TRIS in 50mls distilled water. Add 1M HCl to pH 7.6. Make volume to 250mls

2. *Preparation of stock solution*

   Allow bottle containing DAB tablets to reach room temperature from the freezer. Disslove one tablet in 10ml 0.005M TRIS. Aliquot in 1ml volumes and store at

   -20^{0}C.

3. *Preparation of working substrate*

   For use, thaw out 1ml of stock solution and add 7.5µl freshly prepared 3% H_2O_2 (Stock H_2O_2 30%, so 1:10 dilution made immediately before use).

Preparation of Vectastatin working reagents

<u>Blocking serum:</u>

Add 30µl of stock serum to 2mls TRIS

Biotinylated anti-mouse Ig:

To 1ml of the diluted blocking serum add 5µl of stock biotinylated anti-mouse Ig

ABC reagent

Add 20µl of reagent A to 1ml of TRIS, mix, and add 20µl of reagent B. Mix immediately and leave to stand for approximately 30 minutes before use.

7.2 APPENDIX

SOURCE OF THE EXPERIMENTAL MATERIALS

Poly –L – lysine	Sigma-Aldrich Ltd., Dorset, UK
Cryo-Med mounting medium	Bright Instrument Co. Ltd., Cambridgeshire, UK
ABC Elite Kit	Sigma-Aldrich Ltd., Dorset, UK
DAB (diaminobenzidine)	Dako® Ltd High Wycombe, UK
Erlich's Haematoxylin	BDH Laboratory Supplies, Poole, UK
70% alcohol	Fisher Scientific Ltd., Loughborough, UK
Xylene	BDH Laboratory Supplies, Poole, UK
DPX	BDH Laboratory Supplies, Poole, UK

TNFα(monoclonal mouse)	Dr W G Jiang Department of Surgery University of Wales College of Medicine
CD 3 (monoclonal mouse, clone UCHT1)	Dako® Ltd High Wycombe, UK
CD4 (monoclonal mouse, clone MT310)	Dako® Ltd High Wycombe, UK
CD8 (monoclonal mouse, clone DK25)	Dako® Ltd High Wycombe, UK
CD19 (monoclonal mouse, clone HD37)	Dako® Ltd High Wycombe, UK
CD45 (monoclonal mouse, clone 2B11)	Dako® Ltd High Wycombe, UK
CD68 (monoclonal mouse, clone EBM11)	Dako® Ltd High Wycombe, UK
27e10 (mouse monoclonal, Clone 27e10)	BMA Biomedical AG®, Switzerland

Mannose (monoclonal mouse)	Dr. P Stahl, St. Louis, Missouri, USA
Sialoadhesin(monoclonal mouse)	Dr P Crocker, University of Dundee Dundee, UK
Von Willibrand Factor (polyclonal rabbit)	Chemicon® International Europe Ltd Hampshire, UK
Neutrophil Elastase (monoclonal mouse, Clone AHN-14)	PharMingen International Cowley, Oxford, UK
IL – 6 (monoclonal rat, clone MQ2-6A3)	PharMingen International Cowley, Oxford, UK
Il – 8(monoclonal goat)	R&D Systems, Abington, UK
Il – 10 (monoclonal rat, clone JEs3-19F1)	PharMingen International Cowley, Oxford, UK

MMP9 (polyclonal rabbit)	Calbiochem-Novabiochem©, International, Cambridge, UK
IL1β (monoclonal mouse, clone B1)	R&D Systems, Abington, UK
bFGF (monoclonal mouse, clone 3H3)	Calbiochem-Novabiochem©, International, Cambridge, UK
TGFβ (monoclonal rat, clone A75-2.1)	PharMingen International Cowley, Oxford, UK
VEGF (monoclonal mouse, clone G153-694)	PharMingen International Cowley, Oxford, UK
Anti-goat IgG, Anti-rabbit IgG, Anti-mouse IgG	Sigma – Aldrich Ltd., Dorset, UK