AN INVESTIGATION INTO THE USE OF FIBRIN GLUE AND CULTURED CELLS WITH TISSUE ENGINEERED SKIN REPLACEMENTS

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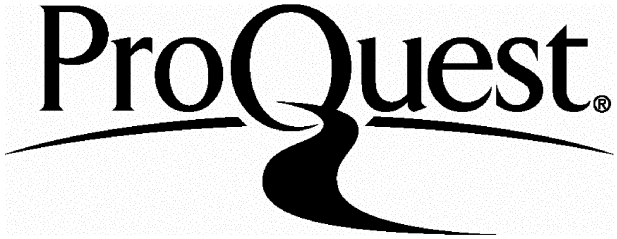

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ABSTRACT

Cultured autologous keratinocytes have been used as an alternative to split skin grafts for patients with major full thickness burns. We have examined the use of cultured autologous keratinocytes with commercially available Integra® artificial skin and Tisseel fibrin glue. The Large White Pig was used as an animal model. Full thickness wounds were isolated in PTFE chambers to prevent wound contraction and marginal re-epithelialisation.

Wounds were grafted with Integra® artificial skin seeded with cultured autologous keratinocytes with or without fibrin glue. The reconstitution of an epidermal cell layer was demonstrated both with or without the use of fibrin glue. There was a trend towards an increased percentage of epidermal cover when fibrin glue was used to seed Integra® (42.1% verses 20.4%) though this was not significant (P=0.097). However, fibrin glue did improve haemostasis and made Integra® application technically easier. Non-cultured autologous keratinocytes produced less epithelium than cultured autologous keratinocytes (6.7% verses 24.6%, P=0.009) and there was evidence of significant differentiated cyst formation within the matrix with the use of non-cultured cells. Cultured fibroblasts seeded into Integra® were shown to persist *in-vivo* for at least two weeks. This was true for both allogenic and autologous fibroblasts. However, the number of fibroblasts seeded into the matrix was insignificant in comparison to the number of fibroblasts derived from local mesenchymal cells.

Spray delivery of cultured keratinocytes is also gaining widespread popularity as an alternative to the use of fragile sheets of keratinocytes. Chambered wounds sprayed with cultured autologous keratinocytes in a suspension of culture medium (without the use of a dermal replacement) were shown to produce a 25.8% epithelial cover by three weeks. When fibrin glue was used as the delivery vehicle for the sprayed cultured autologous cells there was no improvement in the percentage of epithelium at three weeks (P=0.802).

In conclusion, autologous and allogenic fibroblasts seeded into Integra remain viable within the wound for at least two weeks. The use of fibrin glue to seed Integra with cultured autologous keratinocytes produces a good quality surface epithelium. When non-cultured autologous keratinocytes are seeded into Integra, cysts form within the matrix, preventing epithelial formation at the wound surface. The use of fibrin glue for spray delivery of cultured keratinocytes does not increase the quantity of epithelium formed in this model.

CHAPTER 1

INTRODUCTION

1.1 Skin Structure

The skin is the largest organ of the body. It has several homeostatic functions. Skin acts as a barrier to the external environment. It prevents invasion by pathological micro-organisms. It regulates body temperature, controls fluid loss from evaporation, and protects us from ultra-violet radiation. Skin produces vitamin D in the epidermal layer. It provides us with sensation, and also has mechanical properties which allow it to support other tissues, as well as conveying aesthetic qualities and beauty. The skin is able to perform all these functions through a relatively simple structure. In order to replace it we must understand this structure at a macroscopic, microscopic and ultra-structural level.

Skin consists of two layers. The dermis, derived from mesoderm, and the epidermis, derived from ectoderm. The epidermis consists mainly of keratinocytes, but also contains melanocytes, Langerhan cells (immune) and Merkel cells (mechanoreceptor). Keratinocytes undergo a transition from relatively immature cells in the basal layer to more differentiated cells in the outer layers. These differentiated cells produce large quantities of the keratin "family" of proteins and eventually lose their nuclei. The epidermis has been arbitrarily divided into layers. These are the stratum germinativum (the most basal), the stratum granulosum, the stratum lucidum (in which the cells have no nuclei) and the stratum corneum. The stratum corneum consists of dead cells and keratin debris.

Keratinocyte stem cells have an undifferentiated morphology and are slow cycling. However, they have a proliferative reserve which exceeds the life-time of an individual. The stem cells divide producing daughter cells. Some of these replenish the stem cell stocks, whilst others form 'transient amplifying cells' (TA cells)(Wasif et al., 2001). TA cells are rapidly dividing cells which expand clonally until they undergo terminal differentiation into post-mitotic cells. These *in vivo* cell types correspond to holoclones, meroclones and paraclones which have been identified *in vitro* in cell culture studies. Barrandon and Green (Barrandon and Green, 1987) demonstrated that a clone could be assigned to one of three classes. The holoclone has the greatest reproductive capacity, producing colonies of which less than 5% will abort or terminally differentiate. The paraclone, on the other hand, contains only cells with a short replicative lifespan of less than 15 generations.

The precise position of these stem cells in human skin is unclear, but two models exist with good evidence to back them. Firstly, the 'Epidermal Proliferative Unit' model (Potten, 1974). This proposes that a single stem cell lies on the basement membrane at the base of an inverted pyramid of TA cells and post-mitotic cells, which are all derived from that single stem cell. Several studies have supported this theory (Mackenzie, 1997). An alternative model, the 'deep and shallow rete ridge model' has been proposed (Lavker and Sun, 2000) in which the stem cells are thought to only exist in the basal layers of the deep rete ridges. The TA cells in the supra-basal layers of the deep ridges, derived from these stem cells are postulated to provide the basal layers of the shallower

rete ridges. This theory is supported by research based on pulse labeled thymidine pick up by the TA cells, which does not occur in the deep rete ridges.

The epidermis is separated from the underlying connective tissue by the basement membrane. The formation of the basement membrane has been shown to depend on keratinocyte-fibroblast interactions (Timpl, 1996). The structure of the basement membrane is summarised in Figure 1.1. A continuous lamina densa and the formation of hemidesmosomes and anchoring fibrils cannot be identified in co-cultures of fibroblasts and keratinocytes *in vitro* until after 3 weeks of culture.

The predominant type of cell in the dermis is the fibroblast. The fibroblast produces elastin, collagen and other extracellular matrix proteins. The elastin

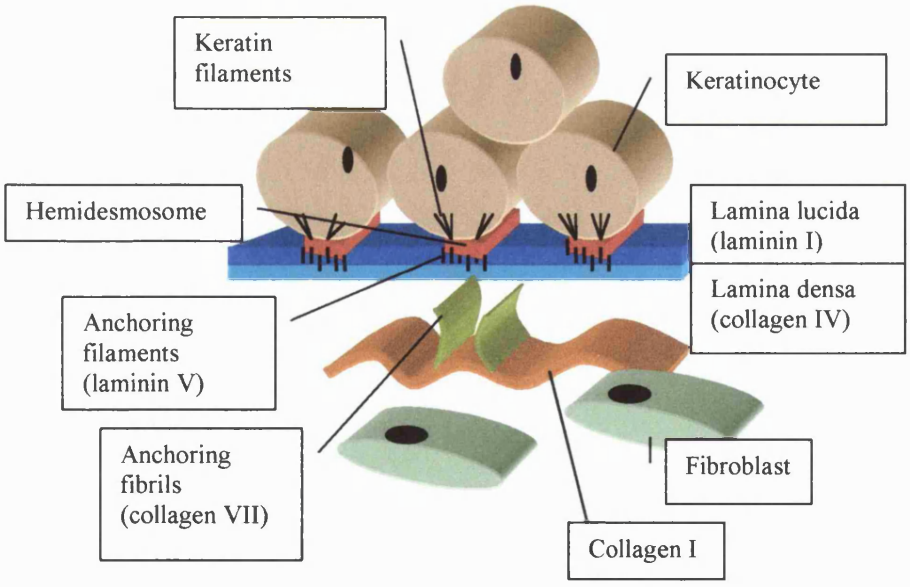


Figure 1.1 – Diagram showing the structure of the basement membrane

and collagen are cross-linked within the semi-fluid ground substance which is composed of macromolecules such as glycosaminoglycan and proteoglycans. The dermis is arbitrarily divided into a superficial papillary and deeper reticular dermis. The reticular layer contains irregular, loosely arranged, coarse elastic fibers interspersed between thick collagen bundles with relatively few fibroblasts and blood vessels compared to the overlying papillary dermis.

1.2 Early wound healing and fibrin glue pathophysiology

Fibrin and fibronectin are initially deposited into wounds from the circulation shortly after injury. A few days after injury fibronectin deposition is continued by wound fibroblasts, macrophages or migrating keratinocytes (Clark, 1993). In response to injury, resident fibroblasts in the surrounding tissue proliferate for the first 3 days and then at day 4 migrate into the wounded site (Hsieh and Chen, 1983). Once within the wound, fibroblasts produce type I procollagen as well as other matrix molecules and deposit these extracellular matrix molecules in the local milieu. Fibroblasts can use a fibrin and fibronectin matrix to move through the wound (Hsieh and Chen, 1983). When exposed to a chemotactic gradient they will migrate along, rather than across the fibronectin fibrils. In this way it can be seen how a greater degree of cross-linking may increase the rate of fibroblast migration through a matrix.

In contrast to normal epidermal cells, wound keratinocytes express functionally active integrin receptors for fibronectin (Toda et al., 1987). Thus, wound keratinocytes can pave the wound surface with a provisional matrix and express cell surface receptors that facilitate their migration across this matrix (Clark,

1990). Fibronectin has been shown to increase the rate of keratinocyte spreading and replication (Weiss et al., 1998) and an increase in the degree of cross-linking may be important, as cross-linking by factor XIII promotes a similar effect. In fact fibronectin binds specifically to fibrin and is covalently cross-linked to the fibrin alpha chain by activated factor XIIIa (Matsuka et al., 1997) which is produced by specific dermal dendrocytes (Sueki et al., 1993). It has also been shown that fibrin will inhibit keratinocyte spreading and replication unless fibronectin is present in vitro (Weiss et al., 1998). This may explain the findings of Shakespeare (Shakespeare and Shakespeare, 1987) who showed that fibrin blocked the attachment of keratinocytes to collagen in vitro.

Fibrin glues are used in clinical practice and work as an adhesive by emulating the exudative phase of wound healing. Early products were made with human fibrin concentrate and thrombin. When the two substances are mixed the thrombin, in the presence of calcium, converts fibrinogen to fibrin (Figure 1.2). A fibrin polymer is formed which has a stable structure that facilitates the growth of collagen-producing fibroblasts (Michel and Harmand, 1990). Further development has lead to the addition of Factor XIII, a fibrin stabilising factor present in blood, and aprotinin, an antiplasmin that will protect the fibrin polymer clot from premature fibrinolysis. Fibrin deposition depends on the relative rates of formation and destruction. Plasmin and other proteolytic enzymes degrade both fibrin and fibrinogen to progressively smaller polypeptide fragments called fibrin degradation products (FDP). Fibrin degradation products in turn inhibit fibrin formation by competitive inhibition of the action of thrombin as well as inhibiting polymerisation of the fibrin

monomers directly. It has been shown that bacteria produce proteolytic enzymes, which can increase the rate of fibrin polymer breakdown directly. These proteolytic enzymes also convert plasminogen to plasmin and thus increase the rate of fibrin polymer breakdown indirectly. Aprotinin blocks this indirect pathway by inhibiting plasmin breakdown of fibrin polymer (Perry et al., 1989).

1.3 Fibrin glue as a template for cellular migration

Wound healing can be modified by changes in cell motility, angiogenesis and modification of matrix production (Naughton and Mansbridge, 1999). Tissue engineered skin implants are designed to optimise these parameters. Changing the fibre size and the fibre spacing can alter the scaffolding properties of cross-linked polymer skin implants. Collagen dermal replacements were shown to have an optimal pore diameter of between 20 and 125 microns (Yannas et al., 1989). This allows optimal cellular in-growth whilst maintaining a dermal scaffold sufficient to retard wound contracture and scarring until cellular colonization has occurred.

A study involving fibrin glue as an implant to promote cellular migration compared pooled donor fibrin glue (60mg/ml fibrinogen) to modified fibrin glue. The fibrin (60mg/mlfibrinogen) was modified by addition of water soluble polymer beads of 100-150 microns to obtain a porous and rough structure of similar pore size. The modified fibrin scaffold increased the volume fraction of fibroblasts and the number of blood vessels compared to a non-modified fibrin scaffold.(Pandit et al., 1998a) The functional mechanisms allowing fibroblasts

to leave the collagenous matrix of normal connective tissue and invade the provisional matrix of the fibrin clot have not been fully defined. It has been demonstrated in vitro that recombinant PDGF at physiological concentrations will stimulate migration of fibroblasts from a collagen gel onto a surface coated with fibrin fibrils (Greiling and Clark, 1997). VEGF, FGF and TGF-β are thought to be important growth factors involved in endothelial cell migration and proliferation in the fibrin matrix (Dvorak et al., 1995;Brown et al., 1996). However, the three-dimensional architecture of the fibrin matrix may be more important for capillary formation (Nehls and Herrmann, 1996).

The rate of implant degradation is an important factor when considering its ability to act as a template for cellular migration and proliferation. Fibrin glue is present in wounds for at least 4 days but is difficult to detect after 10 days (Auger et al., 1993;Xu et al., 1996). The fibrin glue degrades through enzymatic and phagocytic pathways and is therefore an ideal delivery system for cultured cells (Horch et al., 1998) growth factors (Nuytinck et al., 1988) and even antibiotics (Boyce et al., 1994).

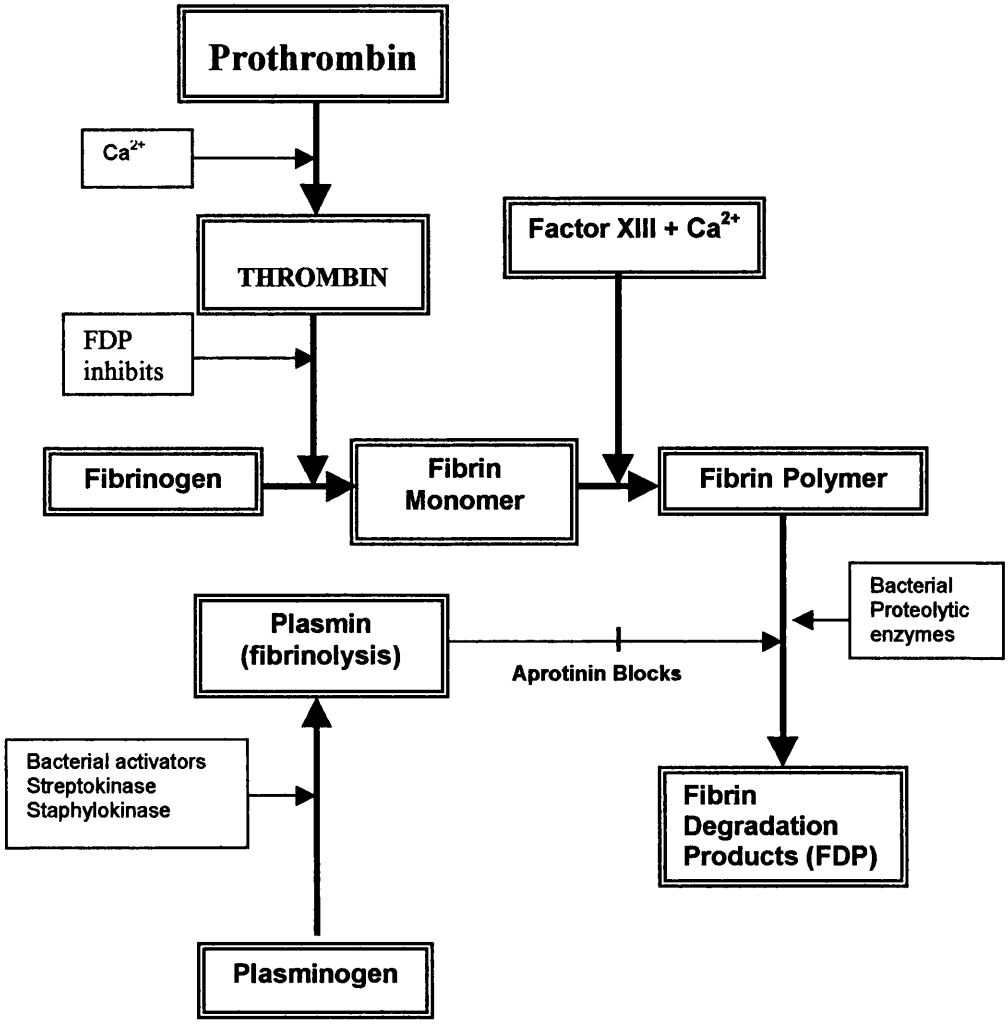


Figure 1.2 - Factors influencing fibrin deposition and breakdown.
Fibrinogen is broken down to fibrin monomer by the action of thrombin. The fibrin monomer then polymerises in the presence of factor XIII and calcium to form a fibrin polymer, which is precipitated as fibrin fibrils in the tissue. The fibrin polymer is broken down to fibrin degradation products by plasmin (which is inhibited by aprotinin) and bacterial proteolytic enzymes.

1.4 Major burn wounds

Burn injuries are classified according to the depth of the injury. Superficial burns involve damage to the epidermis only. They will heal spontaneously with little or no scarring. Partial thickness burns involve the dermis, but not its full depth. They will heal by re-epithelialisation from hair follicle remnants (Miller et al., 1998), but with some scar tissue formation. Full thickness burns involve the whole depth of the dermis. Full thickness wounds will heal by wound contraction if they are small, however, if they are larger they will need to be closed by viable tissue transfer, usually an autologous split skin graft.

The closure of large wounds, when there is limited availability of autologous split skin grafts, has been a significant challenge to burns surgeons over the last century. The mortality from a 46% burn to a 20 year old in 1950 was 50% (Bull, 1954), but by 1991 the mortality from a 70% burn in an equivalent patient was now improved to 50% (Muller and Herndon, 1994) with a much lower mortality for smaller burns. Similarly, a study which examined the clinical course of children admitted for care of acute thermal burns from 1974 to 1980 found a mortality rate of 33.3% for children with 60% to 100% total body surface area burns (Sheridan et al., 2000). Ten years later the mortality rate was 14.3%.

The early debridement of major burn wounds has now become established as the gold standard method of treatment in major burns units (Janzekovic, 1970). The clinical results obtained by primary necrectomies in extensive deep burns

justify an earlier and more adequate treatment in the early postburn phase (Herndon et al., 1989). In a large study involving 11,000 patients in 11 major burns units the speed of full-thickness burn wound closure was found to be associated with mortality rates, lower mortality being associated with quicker wound closure (Wolfe et al., 1983).

The mechanism of improved survival relies on removal of the necrotic tissue. In burned tissue the "zone of necrosis" (Jackson, 1953) contains dead tissue which produces a significant local inflammatory reaction. This local reaction will damage tissue in the "zone of stasis", which contains some tissue capable of regeneration. In massive burns this sequence of events produces a systemic inflammatory reaction, with the release of endotoxins, leukotrienes and prostaglandins. These factors further activate macrophages and neutrophils, which release more inflammatory mediators. The resultant cascade of events leads to systemic shock and multi-organ failure (Oldham et al., 1988;Nuytinck et al., 1988). Removal of the dead tissue prevents this sequence of events.

Improvements in the survival of burns patients due to developments in anaesthesia and intensive care, and the early removal of burned tissue, has highlighted the need for techniques which provide rapid wound closure creating minimal donor sites.

1.5 Skin grafts

A patient with a 50% full thickness burn will have 50% of their normal skin left for harvesting of autologous split skin graft. Autologous split skin graft has

been the gold standard method of burn wound closure for many years, and it is still the technique by which new methods of wound closure are judged. Meshing of split skin grafts allows a donor site to cover a larger area of debrided burn (Davison et al., 1986). However, meshed split thickness skin grafts produce a less satisfactory cosmetic result, as dermis is not replaced in the skin graft gaps, only epithelium. Moreover, split thickness skin grafts are prone to significant contraction and poor quality scar production, especially when compared to full thickness grafts (Sawhney and Monga, 1970).

In major burns that are under 50%, wound closure can often be achieved with available autologous split skin grafts. However, techniques which ensure that graft take is optimised must be used. There is now a strong body of evidence supporting the use of fibrin glue as a useful tool for reducing skin graft loss.

1.6 The use of fibrin glue with skin grafts

The use of fibrin glue for fixing skin grafts has been investigated by a number of authors. The potential benefits can be grouped into three areas: 1) haemostasis; 2) graft adherence and take; 3) antibacterial action.

1.6.1. Haemostasis

Work by Ihara et al in 1984 demonstrated an advantage of fibrin glue for reducing the haemorrhage and improving graft adhesion during burn excision (Ihara et al., 1984). Fibrin glue was used in 10 patients with up to 75% burns who underwent a total of 27 operations. The average transfusion volume per

operation was shown to be 1226ml in comparison to a control group of 2038ml per operation. However no comparison of age or burn size was made between the two groups. The glue was reported to be most effective when used on the limbs under tourniquet. When the tourniquet was released the glue was found to have an effective haemostatic action in comparison to its application on actively bleeding tissue. Several authors have since investigated the effects of fibrin glue on wound haemostasis, and two studies used fibrin glue on graft donor sites as a model (Achauer et al., 1994;Greenhalgh et al., 1999). Auchaur sprayed half the donor site with fibrin glue and thrombin and the other half with thrombin and placebo. No significant difference in the bleeding was found between groups. Likewise Greenhalgh failed to show any improvement in haemostasis of the fibrin treated donor sites by measuring blood soaked dressings. Both groups did notice a subjective reduction in bleeding. A larger study in the USA involving 95 burn victims also investigated the haemostatic effect of fibrin glue (McGill et al., 1997). When used as a topical haemostatic agent to secure skin grafts fibrin glue resulted in an estimated blood loss of $0.5ml/cm^2$ of skin graft, compared with $0.98ml/cm^2$ in the control group (fibrin sealant FS, Baxter Healthcare Corp.).

1.6.2. Graft adherence and take

Autologous skin graft take is inversely proportional to graft thickness and cosmetic and functional success is directly proportional (Teh, 1979). Anything that improves graft take, function and cosmesis with no detrimental effect would be a useful adjunct to surgery. Spangler popularised the use of fibrin glue

in Germany in the early 1970's (Spangler, 1976), and claimed that haemostasis was also obtained by sealing off bleeding surfaces with a fibrin layer. A rat model was used to demonstrate an improved rate of graft take when using fibrin glue (Spangler, Jr. et al., 1973). Fibrin represents a biological system that is completely absorbable, contrary to the commonly used synthetic adhesives. In 1983 Vibe et al (Vibe and Pless, 1983) demonstrate a marginal improvement in split skin graft area take from 83% to 92% with the use of fibrin glue in 20 patients. More significantly he noted an improvement in graft take from 44% to 88% for grafts placed at difficult areas, such as over mobile muscle or close to skin folds. This is a relatively constant finding throughout the literature and shows a slight improvement in simple graft take, but significant improvement in areas that are difficult to graft (Lilius, 1987;Vedung and Hedlung, 1993;Saltz et al., 1989). No authors have demonstrated a detrimental effect of fibrin glue on split skin graft survival, though excessive glue could conceivably reduce nutrient diffusion to the graft.

Several authors have looked at the use of fibrin glue for skin grafting hand burns. The improvement in graft fixation should allow earlier mobilisation, which would result in better functional results with less hand stiffness. Stuart used a single donor adhesive in 16 patients and achieved a 99% graft take (Stuart et al., 1990). Boeckx showed an improvement in two-point discrimination, touch recognition and mobility in a group of 15 patients with dorsal hand burns grafted with fibrin glue compared to a similar control group (Boeckx et al., 1992).

Chemotherapeutic agents such as cyclophosphamide and adriamycin have been shown to alter the permeability of small blood vessels in wounds allowing less fibrin deposition and as a result a reduced incidence of split skin graft take (Ferguson, 1982). Matos showed a reversal of this poor graft take using fibrin glue in a rat model (Matos and Cruz, 1987). Similarly, grafts treated with post-operative irradiation in rats have been shown by immunochemistry and electron microscopy to have a reduction in fibrinogen, fibrin and fibronectin deposition in the wound(Wang et al., 1996).

Burn patients may have an additional benefit from the use of fibrin glue in grafting compared to non-burn patients. It has been shown that partial thickness burns often progress to deeper burns within the first 72 hours of wounding. This is thought to be due to the suppression of fibrinolysis that occurs in burn patients causing progressive clot formation in damaged endothelium around the wound (Fang et al., 1997). Fibrinolytic parameters have been monitored in plasma and burn wound exudate showing that fibrinolysis is activated 2 hours post burn but inactivated at 24 hours until day 10 post injury. Therefore in this time period one might expect fibrin glue to have a longer residency and thus a greater efficacy, although this is yet to be proven in the clinical situation.

Is there any scientific evidence for an improvement in scar formation with the use of fibrin glue in skin grafts? Fibrin glue has been shown to be undetectable on histological and immunohistochemical section by about 14 days (Saltz et al., 1991;Auger et al., 1993). Reduced inflammation in wounds covered by fibrin has been demonstrated in an experimental dog model (Bornemisza et al., 1986),

which may correspond to reduced scarring. Brown *et al* have shown a reduction in skin graft contraction when fibrin glue was used in a rat model (Brown et al., 1992).

1.6.3. Anti-bacterial action

Fibrin glue has been shown to improve skin graft adhesion and take in the presence of bacteria in a rat model (Jabs, Jr. et al., 1992). It was demonstrated that fibrin glue would restore graft adherence to normal levels in graft sites infected with greater than 10^5 bacteria/gm of tissue. Similar results were found in a clinical series looking at fibrin glue use for grafts in infected areas of 23 patients (axilla, perineum and gluteal fold) with favourable outcomes (Vedung and Hedlung, 1993). It was postulated that the beneficial effect might be due to an improvement in phagocyte motility in the fibrin or due to a saturation of the bacterial proteolytic enzymes by the exogenous fibrin. Bacterial growth has been shown to be slower in a clot of fibrin glue than in a physiological clot (Bosche, 1981).

Early skin graft survival depends on stabilisation of the graft by the fibrin network between the graft and the recipient bed. Fibrin glue provides an immediate, highly cross-linked fibrin network that will stabilise the graft and facilitate graft nutrition by serum imbibition (plasmatic circulation) with subsequent ingrowth of vascular buds (neovascularisation). A successful autograft leads to sterilisation of a wound, and the ability of fibrin to stimulate phagocytosis may be an important factor in achieving this (Wood, 1960). Skin graft failure may be due to bacteria causing dissolution of fibrin via proteolytic

enzymes acting on plasminogen to increase the plasmin level as well as acting directly on fibrin polymers (Teh, 1979). *S.aureus*, beta-haemolytic *streptococci* and *P.aeruginosa* have all been found to be associated with high levels of FDP when detected in wounds. Their presence is followed by a delayed rise in wound plasmin activity, and a reduction in fibrinogen levels with subsequent graft loss. Although the bacterial load is an important factor in graft survival, the ability of certain bacteria to produce proteolytic enzymes may have more significance.

Teh found that attempts to increase the fibrin deposition artificially (by addition of thrombin or fibrinogen, or measures to prevent the digestion of fibrin by inhibiting the action of plasmin and proteolytic enzymes) had no effect on graft survival (Teh, 1979). Conversely, Perry et al found that fibrin clot dissolution was enhanced by addition of plasminogen to bacterial culture with a rise in the FDP levels of the supernatant (Perry et al., 1989). Both aprotinin (a plasmin inhibitor) and epsilon-aminocaproic acid (an inhibitor of plasminogen activators) were capable of reducing clot destruction by bacteria.

1.7 Types of fibrin glue

In its simplest form fibrin glue can be made by spinning down 50mls of a patient's blood in a citrated tube and removing the plasma supernatant, which contains fibrinogen. Plasma can be added to a solution of thrombin and calcium to form fibrin on a wound bed (Buckley et al., 1999). Plasma prepared in this way has a relatively low fibrinogen content. It has been shown that increasing the fibrinogen concentration of the glue increases the shear adhesive strength

(Saltz et al., 1991;Sierra et al., 1992). Fibrinogen concentration can be increased in two ways, either by concentrating the fibrinogen taken from a single donor, or by pooling the fibrinogen taken from many donors.

The majority of commercially produced fibrin glues e.g. Tisseel, Beriplast, etc (see Table 1) consist of pooled cryoprecipitated fibrinogen and fibronectin combined with factor XIII and aprotinin, which are added to thrombin and calcium. Thrombin and Factor XIII promote fibrin and fibronectin cross-linking and promote its adherence to wound collagen, while aprotinin reduces plasmin breakdown of the fibrin polymers formed. These products have been used extensively in Europe and have recently been given FDA approval in America. Pooled blood products obviously carry a risk of disease transmission, particularly Hep B and more recently HIV. Donor screening, heat-treating and the use of a solvent/detergent suspension for inactivation of lipid-enveloped blood-borne viruses in plasma derivatives (Horowitz et al., 1988) has made these products safer. Alternatively single donor products improve the safety margin. A multicenter trial to evaluate the safety of pooled human fibrin sealant (FS) for the treatment of burn wounds showed no rise in viral titres for HIV, Hep A, B, C, epstein-barr, or CMV(Greenhalgh et al., 1999). Wilson et al recently reviewed all published data relating to the spread of variant CJD by blood products (Wilson et al., 2000). It was concluded that there was no evidence to suggest any risk of CJD transmission via blood transfusion. However some commercial products contain bovine aprotinin and thrombin and there is a theoretical risk of variant CJD from these sources. Autologous fibrinogen preparations completely avoid the risk of viral transmission and

would be inherently safer, provided that no exogenous thrombin or aprotinin are used.

Several methods have been described to isolate autologous fibrinogen. To be of clinical use the technique must be relatively simple, quick and preferably inexpensive. Saltz et al used a cryoprecipitation technique, which took about two days to complete, and required around 300mls of plasma (Saltz et al., 1991). Two cycles of freezing to -18°C produced a fibrinogen concentration of approximately 45mg/ml. In a previous paper these authors achieved a concentration of 6mg/ml with a single freeze cycle. A much faster method has been described using ammonium sulphate (Chakravorty and Sosnowski, 1989), or ethanol (Dahlstrom et al., 1992) to precipitate out the fibrinogen. These methods produced a fibrinogen level of around 20mg/ml and required only around 50mls of blood to produce up to 5 mls of glue. These methods of fibrinogen isolation did not achieve the bonding strength of commercial pooled fibrinogen products, which contain between 70-100mg/ml fibrinogen.

An automated system for producing 5 mls of autologous fibrin sealant from 120mls of blood in 30 minutes has been developed by ConvaTec (Skillman, NJ). The Vivostat™ system (Kjaergard et al., 1997;Kjaergard and Trumbull, 1998) relies on biotin-batroxobin which catalyses the release of fibrinopeptide A from fibrinogen and does not activate factor XIII. This results in the formation of a fibrin I polymer which is acid soluble. Fibrin I polymer is then isolated after removing the biotin-batroxobin with avidin-agarose. The acid solution containing fibrin I polymer is neutralised with alkaline bicarbonate by

co-spraying the two solutions onto the wound. On the wound surface, in the presence of calcium ions, endogenous thrombin cleaves fibrinopeptide B from fibrin I to form fibrin II. Thrombin also activates factor XIII which acts on the fibrin II polymer to form a stable fibrin II polymer which acts as a glue

Most commercial pooled fibrin glue products and products based on autologous fibrinogen contain thrombin for the activation of the fibrinogen. Initially bovine thrombin was used, but several cases of anaphylaxis to the bovine thrombin have been reported (Rothenberg and Moy, 1993). Antibody formation to bovine factor V (contained in bovine thrombin preparations) subsequently causing depletion of human factor V in several patients has also been described resulting in morbidity and mortality (Cruickshank et al., 1994). Human thrombin preparations are now widely used as an alternative, though some commercial products still contain bovine aprotinin.

1.8 Tissue engineered dermal replacements

In major burns over 50%, wound closure cannot be rapidly achieved with split skin grafts. Donor sites need to regenerate new epithelium, and new techniques are needed to temporarily close wounds while this happens. This can be achieved with cadaveric allograft. The pathological immunosuppression present in the early stages of a severe burn injury protects allografts from rejection during this period. Cadaveric material is supplied by skin banks where it is frequently treated by cryopreservation (Greenleaf and Hansbrough, 1994;Bravo et al., 2000). The potential for disease transmission is a significant factor and careful screening of prospective donor material is needed to reduce the risk of

transmission of infective agents. However, this does not reduce this to zero (Pirnay et al., 1997). Cadaveric allograft only produces a transient means of regaining wound closure, and it is eventually rejected. This has prompted the need for an alternative permanent wound closure material.

In the past 25 years, significant biotechnological advancements have been made in defining the criteria and manufacturing ingredients in materials that could serve as skin replacements for permanent wound closure. The optimal skin replacement should have the functional and cosmetic properties of the dermis and the epidermis. It should provide rapid wound coverage and barrier protection to micro-organisms, normalise fluid flux and hypermetabolism, and provide long-term stability without contraction or hypertrophic scarring. In addition, the optimal skin replacement should be non-toxic, easy to store and use, and cost-effective.

Wound closure requires a material to restore epidermal barrier function and become incorporated into the healing wound (Tompkins and Burke, 1996), whereas materials used for *wound cover* rely on in-growth of granulation tissue for adhesion. The ultimate aim of the burn surgeon is to produce wound closure, in the fastest possible time, with the optimal cosmetic result. Unfortunately no product has yet been developed which performs this function better than autologous split skin graft when it is available.

Since 1975 it has been possible to cultivate human keratinocytes *in vitro* using lethally irradiated 3T3 mouse fibroblasts in a specific culture medium.(Rheinwald and Green, 1975). It is now possible to culture enough

keratinocytes over a 5-week period to cover the entire body surface of an adult with sheets of autologous keratinocytes several cells thick. This was a significant advance in the treatment of patients with major burns, with one study showing a reduction in mortality from 48% to 14% associated with its use (Munster, 1996). However, sheets of autologous keratinocytes have several drawbacks in clinical use. A relatively unstable epidermis is produced which is often prone to blister on minor trauma (Desai et al., 1991). Keratinocytes take a significant time to cultivate, during which the burn patient's condition may deteriorate. The first keratinocytes are typically ready three weeks after the biopsy is taken. Surgical "take" of the sheets is relatively poor, ranging from 15% to 70%(Munster, 1992;Rue, III et al., 1993;Gallico, III et al., 1984;Compton et al., 1989), and it is now commonly accepted that the quality of epidermis can be improved by providing a dermal layer for skin replacements

An allogenic cadaveric dermis can be used to provide this dermal replacement (Cuono et al., 1986); (Compton et al., 1993). Cuono used allograft to close full thickness burn wounds, but when it had "taken" he debrided the allogenic epidermis and replaced this with cultured epithelial autograft. Other authors have used a non-immunogenic modified dermis. AlloDerm® is processed human cadaver skin from which the epidermis has been removed and the cellular components of the dermis extracted prior to cryopreservation in order to avoid a specific immune response (Wainwright et al., 1996). Following application to a wound bed, it is repopulated by host cells, revascularised and incorporated into the tissue. Its role is as a template for dermal regeneration. It is reported to have good take rates and reduce subsequent scarring to full

thickness wounds, while allowing grafting of an ultra-thin split skin graft as a one-stage procedure.

Integra® Artificial Skin (Integra Life Science Corporation, Plainsboro, New Jersey) is a synthetic dermal substitute developed for use in burns patients. It was originally described by Yannas (Yannas and Burke, 1980;Yannas et al., 1980), and has now become one of the most widely accepted dermal replacements in clinical use. Integra® has a bilaminar structure, consisting of a cross-linked bovine collagen and glycosaminoglycan matrix, coated on one side with a silicone membrane that functions as a temporary synthetic epidermal replacement. The collagen layer forms a vascular "neodermis", a process that takes approximately 3 weeks in humans. The patient's own endothelial cells and fibroblasts migrate into the matrix through 70-200μm pores. Smaller pores delay migration and prevent bio-integration, whereas larger pores provide insufficient attachment area for invading host cells. The silicone layer is conventionally removed after 3 weeks, and an ultra thin split skin graft applied.

Integra® "take" rates are variable and are dependant on operator experience. A trial involving 106 patients from several centres found the median "take" was 85%, compared to take rates of 95% in the controls using split skin grafts (SSG) (Heimbach et al., 1988). This trial also highlighted the improved cosmetic results, not only of the Integra®, but also of the donor sites. The split skin grafts needed to resurface the Integra® can be harvested at 0.15mm, rather than the average split thickness skin graft which is 0.33mm thick. These were shown to heal four days sooner with less hypertrophic scarring.

Long term results of Integra® use are now emerging. A study at the Massachusetts General Hospital and the Boston Unit of the Shriners Burns Institute, which included 121 patients with follow-up as long as ten years, reports successful engraftment rates of over 80% (Sheridan et al., 1994). Hypertrophic scar formation was also reduced with 93% having absent or minimal hypertrophic scarring. All patients had excellent function of involved joints. Interestingly, areas of Integra® grafted in children appeared to grow with the child. The general opinion of the patients was that the areas grafted with Integra® artificial skin were cosmetically superior to those where split skin autograft was used alone, although in no instances was it felt to be identical to normal skin.

Integra® requires a two-stage procedure necessitating a minimum time interval of three weeks between the application of the Integra and the SSG to allow neodemis formation. The use of Integra® in combination with cultured epidermal autografts is a very attractive proposition. The three-week time delay would allow for expansion of a skin biopsy into cultured epidermal sheets. However, there are very few reports of the successful combination of cultured epidermal autograft sheets with Integra® in clinical use for reasons that are as yet unclear (Pandya et al., 1998).

Integra® is relatively expensive when compared to cadaveric allograft skin from skin banks and there is a reported steep learning curve with initial high failure rates. The advantages are that it provides improved elasticity and cosmesis to an ultra thin SSG, with reduced donor site morbidity compared to a standard

thickness SSG. It is available immediately and is without the risks of cross infection related to allograft. Integra® has an important role in providing immediate wound cover following early excision for patients with insufficient autograft. However, a new clinical role is emerging for the use of Integra® to resurface secondary burn wound deformities, such as neck contractures (Hunt et al., 2000) and upper limb contractures (Chou et al., 2001).

1.9 Tissue engineered epidermal replacements

Cultured keratinocyte sheets are available commercially from a number of companies eg Epicel in the (Genzyme Tissue Repair Corporation, Cambridge, MA.), and are also a relatively straight forward undertaking for suitably equipped university or hospital laboratories. However, they are expensive, with a time delay of between three to five weeks to achieve sufficient to cover an adult. Several alternatives have been investigated.

1.9.1. Allogenic keratinocytes

The use of pre-grown allogeneic keratinocytes has been extensively investigated in an attempt to overcome the problem posed by the time delay in growing confluent autologous keratinocytes for wound closure. Allogeneic keratinocytes sheets are not acutely rejected and improved healing following application of allogeneic keratinocytes has been demonstrated (Phillips and Gilchrest, 1991). However, Y chromosome and DNA probes have shown that allogeneic cells survive less than one week when grafted onto tattoo excision wounds or ulcers (Brain et al., 1989;Phillips et al., 1990), but over six weeks when applied to a

split skin graft donor site (Thivolet et al., 1986;Zhao et al., 1992;Eisinger et al., 1988). The improved wound healing that is often observed or at least claimed, has therefore been attributed to the secretion of growth factors and cytokines by these cells.

Allogenic keratinocytes form a temporary epidermis which provides wound closure and the secretion of growth factors and cytokines while the allogenic keratinocytes are progressively replaced (Gielen et al., 1987). Cultured allogeneic keratinocytes are therefore regarded as materials for wound cover, since they will not in themselves achieve wound closure.

1.9.2. Sub-confluent autologous keratinocytes

Culturing cells to a non-confluent state *in vitro*, and then delivering them to the wound in suspension can reduce the time needed to cultivate epithelial sheets. This has the advantage of providing cells that have not undergone phenotypic changes associated with contact inhibition and should therefore have undiminished adhesive and proliferative potential. It also avoids the use of the proteolytic enzyme Dispase® that is used to release epidermal sheets in conventional keratinocyte technology. Dispase® may reduce the surface antigen expression of the keratinocytes and reduce their adhesive potential (Myers et al., 1997).

Many groups are working on the development of keratinocyte delivery systems in the hope that this may reduce the costs and improve the take and the quality of the resulting epidermis. In order to deliver sub-confluent keratinocytes to a

wound bed, they must either be dispersed as a suspension in a suitable medium, or be attached to some form of transferable substrate. Keratinocytes have been attached to hyal uronic membranes, (Myers et al., 1997) the polyurethane wound dressing Hydroderm® (Rennekampff et al., 1996), and a polymer membrane (EpiGen®)(Barlow, 1992). These systems are generally regarded as research materials awaiting further investigation. However, one delivery system which has been used extensively both *in vitro* and *in vivo* studies is fibrin glue.

1.9.3. Fibrin glue as a delivery system for cultured keratinocytes

Hunyadi reported the first use of fibrin glue with cultured keratinocytes in 1988. It was demonstrated that fibrin glue (Beriplast) could be used to effectively deliver autologous keratinocytes mixed with the fibrinogen component to the wound. A marked increase was found in the rate of leg ulcer healing compared to a control group (Hunyadi et al., 1988).

Ronfard et al developed a technique in 1991 for culturing sheets of autologous keratinocytes on fibrin glue and reported its use in two burn patients. The only difference to conventional techniques was that the last subculture prior to grafting was set up on a petri dish coated with fibrin glue (Biocol) and seeded with irradiated 3T3 mouse fibroblasts that are a standard component of keratinocyte culture (Ronfard et al., 1991). It was found that in vitro there was no destruction of the fibrin matrix for up to 15 days, probably due to the aprotinin in the product. The autologous keratinocytes could then be transferred to the patient on the fibrin sheets alleviating the need to use Dispase®. Inverted fibrin sheets were used on all but one area, with the keratinocytes closest to the

wound. The worst graft take was on the non-inverted area, and in areas the graft had been inverted, no apparent alteration of anchorage or growth of the keratinocytes was found on histological analysis. The technique was claimed to accelerate the standard process of handling fragile cultured keratinocyte sheets and permitted the use of subconfluent cells.

The benefit of using fibrin glue to secure traditionally produced sheets of cultured keratinocytes was described in an athymic mouse model (Xu et al., 1996). Fibrin glue (Hemaseel) was sprayed onto the wound bed prior to deposition of the epidermal sheets and compared to a control group in which no fibrin glue was used. Seven days after transplantation the percentage of graft take over the total surface area grafted was greater in animals that had received the tissue sealant application, compared to controls. No difference was found 14 and 21 days post-grafting. In contrast, the percentage of graft take over the bony area (spinal) was significantly increased in animals grafted with previous application of sealant compared to controls at 7, 14 and 21 days post-grafting. Immunohistological and ultrastructural analysis showed that the evolution of the cultured human epidermis after transplantation was similar in both groups. The basement membrane was well-structured 21 days after transplantation. The sealant was present at 4 days but not at 21 days post-grafting. Therefore, it was concluded that the application of fibrin sealant before cultured epidermal sheet deposition on a nude mouse graft bed is innocuous and enhances their mechanical stability. Auger et al showed a 20% improvement in cultured epithelial graft take in a similar model using Tisseel fibrin glue (Auger et al., 1993).

Fibrin glue has also been used to deliver cultured human keratinocytes to a wound in suspension. In vitro studies showed keratinocytes to remain viable in suspension in fibrin for at least 5 days (Jiao et al., 1998). When compared to standard cultured epidermal sheet grafts in a nude mouse model, reepithelialisation was similar but reconstitution of the dermo-epidermal junction zone, as shown by electron microscopy and immunohistochemistry was significantly enhanced by the fibrin-glue suspension technique (Horch et al., 1998). It was concluded that the fibrin glue not only delivers highly proliferative keratinocytes but also provides an optimal milieu for their migration, proliferation and differentiation.

The same authors have used cultured autologous keratinocytes suspended in fibrin glue (KFGS) with allogeneic skin overgraft in several burn patients (Kaiser et al., 1994;Horch et al., 1994;Stark and Kaiser, 1994). Cultured cells which are 70% confluent prior to trypsinisation were mixed with the fibrin component of the fibrin glue (Tissucol), and the cell-containing suspension was used to secure the allograft skin to the debrided burn wound. The allogenic epidermis underwent immunological rejection and cultured autologous keratinocytes replaced them. The fate of the allogenic dermis was less clear. Histological evidence indicated integration of allodermis into the wound, although it was stated that further immunohistochemical studies were needed to verify this finding. The Vivostat^{TM} system has been used in a similar way in an animal model to deliver sub-confluent cultured autologous keratinocytes to a wound in an autologous fibrin spray (Grant et al., 1998).

Fibrin gels have been used as a "dermal matrix" containing fibroblasts, keratinocytes or a combination of the two. Meana et al created a fibrin gel by adding 3ml of fibrinogen cryoprecipitate to 12ml of DMEM 10% FCS containing $0.5x10^6$ human fibroblasts and bovine aprotinin (Meana et al., 1998). This was combined with thrombin and calcium to form a gel and then seeded with cultured human keratinocytes. Keratinocytes would not proliferate efficiently without the human fibroblasts in the matrix. These gels were successfully grafted on to athymic mice. Stains for the two basement membrane proteins, type IV collagen and laminin were only positive when both fibroblasts and keratinocytes were used.

Pellegrini et al showed that keratinocytes cultured on fibrin glue maintained the relative percentage of holoclones, meroclones and paraclones, proving this fibrin technique does not induce clonal conversion and consequent loss of epidermal stem cells (Pellegrini et al., 1999). Fibrin glue cultured keratinocyte autografts bearing stem cells applied "cells-up" to massive full-thickness burns (initially treated with allo-dermis) displayed a high keratinocyte take rate, which was reproducible, permanent and maintained long-term proliferative potential.

A recent paper reported a system for the cultivation of keratinocytes on acellular human dermis using fibrin glue and 3T3 feeder cells, hence avoiding the need for a two stage procedure to create a dermal and epidermal component (Lam et al., 2000). De-epithelialised dermis was used in which the fibroblasts were inactivated by repeated freeze-thaw cycles. Keratinocytes were delivered to the dermis and then fixed in position with a fibrin glue spray. This complex

was cultured at the air liquid interface in a medium, which contained 3T3 fibroblasts. It has yet to be proven whether this in vitro product works in a clinical situation.

1.10 Tissue engineered skin substitutes

Autologous keratinocyte sheets or sub-confluent cells take time to re-establish a new basement membrane. It is this structure which is the key to a robust and adherent epidermal barrier. The obvious solution to this problem is to allow the epithelium to form a basement membrane during the culture process and to keep this intact during cultured epidermal autograft delivery. This can be achieved by growing the cells on some kind of biomaterial. This approach is often referred to as a "composite" in skin replacement technology. The quality of the basement membrane can often be improved by co-culture of fibroblasts and keratinocytes on or within the biomaterial. Human keratinocytes or fibroblasts cultured separately express very low levels of collagen VII, the major structural protein of the anchoring fibrils in the basement membrane. However, collagen VII expression is greatly stimulated when fibroblasts and keratinocytes are co-cultured (Konig and Bruckner-Tuderman, 1991).

1.10.1. Skin substitutes for wound "cover"

Several commercially available Skin substitutes have been developed for wound cover. However, they are composed of allogenic cells which will not produce wound closure. TransCyte™ (Advanced Tissue Sciences, Inc. La Jolla, California, USA) consists of collagen coated nylon mesh seeded with neonatal

fibroblasts in order to improve its healing properties. As nylon is not biodegradeable this material can not act as a dermal substitute. Hansbrough (Hansbrough et al., 1997) performed a clinical trial comparing the use of cryopreserved allograft to TransCyte™ as a temporary dermal analogue in 10 patients, though TransCyte™ is probably more correctly termed a temporary wound cover than dermal analogue. The wounds were closed with meshed split skin autograft. The results showed that autograft adherence and take were at least as good after TransCyte™ as with allograft. In a larger multi-centre trial with 66 patients it was noted that as well as showing good adherence, TransCyte™ was easier to remove resulting in less bleeding than allograft (Purdue et al., 1997). Histologically a comparison of TransCyte™ to allograft in burn wounds showed the only significant difference to be increased granulation tissue with allograft (Spielvogel, 1997), which could indicate better cosmesis after TransCyte™. Pediatric burns greater than 7% total body surface that underwent wound closure with TransCyte™ have been shown to subsequently require a lower percentage of split skin autograft, compared to standard therapy consisting of application of antimicrobial ointments and hydrodebridement (Lukish et al., 2001).

Apligraf® (Organogenesis Inc, Canton, MA and Novartis Pharmaceuticals Corporation, East Hanover, NJ) consists of a type I bovine collagen gel impregnated with living neonatal allogeneic fibroblasts, with an overlying cornified epidermal layer of neonatal allogeneic keratinocytes. It appears to hasten healing particularly in the deeper and more chronic wounds (Falanga,

2000), though little experience exists of its use in burns surgery. The potential of Apligraf® to improve cosmetic and functional outcomes when applied over meshed split thickness autografts has been evaluated in a multicenter, randomized controlled clinical trial (Waymack et al., 2000). There was no difference in the percent take of autograft in the presence or absence of Apligraf®. However, in the Apligraf® group the cosmetic result in terms of pigmentation, pliability and vascularity of the skin graft was significantly better than in the control group.

Dermagraft® (Advanced Tissue Sciences, Inc. La Jolla, California, USA) is a cryopreserved living dermal structure, manufactured by cultivating neonatal allogeneic fibroblasts on a biodegradable polymer scaffold (polyglycolic acid or polyglactin-910, marketed as Dexon™ or Vicryl™ respectively) (Hansbrough et al., 1992). The fibroblasts become confluent within the polymer mesh, secreting growth factors and dermal matrix proteins (collagens, tenascin, vitronectin and glycosaminoglycans) thus creating a living dermal structure (Hansbrough et al., 1994). This remains viable and metabolically active after implantation into the wound, despite cryopreservation (Cooper et al., 1991). Although Dermagraft® has not been used extensively for burns it has been used as a dermal replacement beneath meshed split skin grafts on full thickness wounds (Hansbrough et al., 1992). The take rate of the SSG was comparable to grafting on the wound bed alone. Wound histology showed extrusion of vicryl fibres from the wound although this was not clinically apparent. Further studies will be needed to determine any long-term benefits following the application of Dermagraft® under meshed split skin grafts.

1.10.2. Skin substitutes for wound "closure"

Autologous human dermal-epidermal composites based on sterilized human allodermis have been designed for clinical use (Chakrabarty et al., 1999). These composites were formed from standardised glycerolised or improved ethylene oxide treatment allograft, pre-seeded with fibroblasts, then seeded with keratinocytes. Early in vitro results have been promising though clinical trials are awaited.

In vitro studies placing sub-confluent human keratinocytes on the surface of a collagen-GAG matrix, similar to that used in Integra®, showed good attachment and formed a confluent epidermis (Boyce and Hansbrough, 1988). The addition of autologous fibroblasts to the matrix has allowed stratified layers of cultured autologous keratinocytes to form *in vitro*. Autologous keratinocytes cultured with autologous fibroblasts within the matrix improved basement membrane formation, increased production of laminin and type IV collagen and produced thicker epithelial layers in vitro. (Cooper et al., 1991). Clinical studies have demonstrated the formation of a basement membrane within nine days.(Hansbrough et al., 1989). Cultured skin substitutes (CSS) consisting of human keratinocytes and fibroblasts attached to collagen-glycosaminoglycan substrates have now been tested in athymic mice showing rapid wound closure (Boyce, 1998). More recently in burn patients, CSS have been successfully applied to vascularised Integra® Artificial Skin for the closure of excised full-thickness wounds (Boyce et al., 1999).

The structure, composition and cost of commercially available dermal, epidermal and composite products are summarised in Figure 1.3.

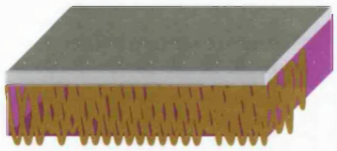
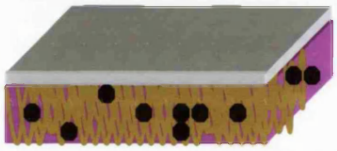
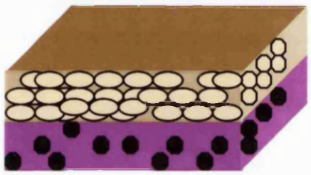
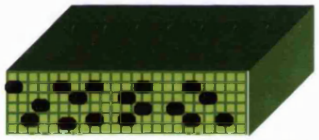
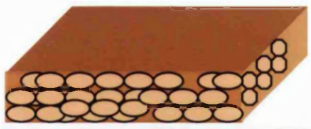
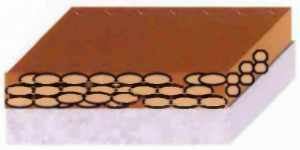
Trade Name	Schematic Representation	Layers	Cost	Cost per cm^2
Biobrane™ (Dow Hickam/Bertek Pharmaceuticals, Sugar Land, TX).		1. Silicone 2. Nylon Mesh 3. Collagen	5x5cm £12.00	£0.48
Transcyte® (Advanced Tissue Sciences, Inc. La Jolla, California, USA)		1. Silicone 2. Nylon Mesh 3. Collagen seeded with neonatal fibroblasts	13x9cm £921.15	£7.87
Apligraf® (Organogenesis Inc, Canton, MA and Novartis Pharmaceuticals Corporation, East Hanover, NJ)		1. Neonatal keratinocytes 2. Collagen seeded with neonatal fibroblasts	7.5 cm diam disc £626.00	£14.20
Dermagraft® (Advanced Tissue Sciences, Inc. La Jolla, California, USA)		Polyglycolic acid (Dexon™) or polyglactin-910 (Vicryl™) seeded with neonatal fibroblasts	5x7.5cm £267.80	£7.14
Integra® (Integra Life Science Corporation, Plainsboro, New Jersey)		1. Silicone 2. Collagen and glycosaminoglycan	10x25cm £830.00	£3.32
Alloderm® (LifeCell,Woodlands, Texas.)		Acellular de-epithelialised cadaver dermis	4x12cm £283.00	£5.90
Epicel™ (Genzyme tissue repair corporation, Cambridge, MA.)		Cultured autologous keratinocytes	Not yet licensed in UK	
Cadaveric allograft (from not for profit skin banks)		• cryopreserved in order to retain viability • lyophilised • glycerolised	similar price for all three various sizes	£0.60

Figure 1.3 - A guide to biological skin substitutes. The first column denotes the product name and its manufacturer. The second column is a schematic representation of the components of the product, with the contents of each layer described in column three. The final two columns quote the cost of the product at the time of writing this review.

1.11 Wound contraction and scar contractures

Wound contraction is the mechanism by which the edges of an open wound come together as a result of forces from within that wound. This is not the same as scar contracture, which is the shrinkage of a closed wound. However, the two are clearly related. The development of hypertrophic scars has been shown to correlate with the time a wound takes to heal (Deitch et al., 1983). If a wound takes more than 21 days to heal it will have a 78% chance of developing a hypertrophic scar prone to wound contracture. Clearly techniques which involve wound closure, rather than wound cover, will have a favourable outcome.

The reduction in the size of a surgical wound over time is also dependent on the surgical procedure used to treat that wound. An open wound will contract faster than a wound treated with a skin graft (Sawhney and Monga, 1970). The contraction of a wound covered with a skin graft depends on the percentage of the dermal component of the graft, rather than the thickness of the graft (Corps, 1969), and a skin flap will prevent contraction of a wound to a greater extent than a skin graft (Baran and Horton, 1972). One study has shown that wounds covered with full thickness skin grafts contract by 44.9%, whilst equivalent wounds covered in split thickness skin grafts contract by 69.9% (Sawhney and Monga, 1970). A wound covered with an expanded meshed split skin graft contracts more than with a sheet split skin graft (Petry and Wortham, 1986). Meshing a skin graft has no effect on the rate of wound contraction if the meshed graft is not expanded (Fifer et al., 1993).

On the cellular level, two main theories exist to explain the phenomenon of wound contraction. The first proposes that the myofibroblast is responsible for the contractile force. Myofibroblasts are cells which show morphological and biochemical features of both fibroblasts and smooth muscle cells. First demonstrated in rat granulation tissue (Majno et al., 1971), they can now be identified using monoclonal antibodies to α-smooth muscle (Skalli et al., 1986). Electron microscopy has demonstrated that they contain thick cytoplasmic fibril bundles (stress fibres) and cell to cell contacts similar to hemidesmosomes (Gabbiani et al., 1972). It is believed that these structures allow contraction and subsequent rearrangement of the surrounding connective tissue.

The second popular theory proposes that fibroblasts exert a contraction force by extending and then retracting filopodia, rather than by contraction of their entire cytoskeleton. This explains the observation that there is poor correlation between the presence of myofibroblasts and wound contraction (Darby et al., 1990). Other studies have shown that stress fibers are not always present in a contracting wound (Ehrlich, 1988). Myofibroblasts may appear after the dynamic contraction phase and have a role in maintaining tension within the tissue while the extra-cellular matrix is remodelled. In fact α-SMA expression may be associated with terminal differentiation of proliferative wound fibroblasts prior to apoptosis. Accelerated maturation of a closed wound into a mature scar has been shown to be associated with a rapid reduction in the myofibroblast population, which appears to be due to an increase in the apoptotic cell death (Desmouliere et al., 1995). The process of normal wound repair after tissue injury follows a closely regulated sequence including the

activation and the proliferation of fibroblastic cells, and their subsequent differentiation into myofibroblasts. The control of this process is probably related to the cytokine profile, the extra-cellular matrix components and cell to cell interactions. In tissue flaps the maximum number of myofibroblasts are present after 2 days (Garbin et al., 1996). This compares with the maximum number of myofibroblasts being present in granulation tissue at 10 days. This correlates with the observation that there is a decrease in the rate of contracture formation in burns scars which are released and resurfaced with free flaps (Ohmori, 1982). Similarly, myofibroblast populations have been shown to decrease much more rapidly in wounds covered with full thickness skin grafts compared to wounds covered with split skin grafts (Rudolph, 1979). This may explain the observation that full thickness skin grafts appear to reduce myofibroblast proliferation in dupytrens disease and reduce recurrence rates (Rudolph and Vande, 1991). In pathological situations, the normal resolution stages are abolished and the proliferation of myofibroblasts continues, inducing excessive accumulation of extracellular matrix. The differentiation of fibroblastic cells into myofibroblasts is an early event in the development of tissue fibrosis for many fibroproliferative disease states (Cassiman et al., 2002;Yang and Liu, 2002;Morishima et al., 2001).

Keloids and hypertrophic scars are the dermal equivalent of these fibroproliferative disorders. TGF-β has been shown to enhance collagen fibronectin and proteoglycan synthesis by wound fibroblasts (Ignotz and Massague, 1986), as well as reducing matrix destruction by increasing collagenase inhibitors (Overall et al., 1989). TGF-β has been identified *in vivo*

in a number of fibroproliferative diseases, including scleroderma (Kulozik et al., 1990), pulmonary fibrosis(Khalil et al., 1996) and glomerulonephritis (Border and Noble, 1998;Peters et al., 1997). Increased levels of TGF-β messenger RNA have been found in hypertrophic scars following thermal injury (Ghahary et al., 1993), and fibroblasts cultured from hypertrophic scars, keloids and dupytrens nodules have all been shown to respond with enhanced collagen protein synthesis to exogenous TGF-β when compared to control wound fibroblasts (Younai et al., 1994;Badalamente et al., 1992). Burns patients with severe hypertrophic scarring have been demonstrated to have increased levels of serum TGF-β (Polo et al., 1997). It therefore seems feasible that an imbalance in the production of cytokines by immune cells may be the mechanism responsible for proliferative scar formation in burns patients. Alpha-interferon, which down regulates the effect of TGF-β on fibroblasts, has been used to treat hepatic(Serejo et al., 2001) and pulmonary fibrosis (Kamisako et al., 1993). More recently it has been used in a clinical trial on burns patients with hypertrophic scarring, with improvement in seven out of nine patients (Tredget et al., 1998).

The extracellular matrix of hypertrophic scars also contains the large proteoglycan versican which binds water and attracts it into the scar, as well as having an abnormally high type III collagen content. The small proteoglycan decorin may also be important in the formation of hypertrophic scars. Decorin has a region in its protein core which binds TGF-β and neutralizes it (Yamaguchi et al., 1990). The appearance of decorin in wounds of burns patients with hypertrophic scarring has been shown to occur 12 months after

wounding (Sayani et al., 2000). This is significant as this is the time after wounding that hypertrophic scar resolution is considered to begin.

1.12 Retro-viral transduction of cell markers

The potential benefits in the use of cultured cells for skin replacement is often unclear. How long cultured cells remain within a wound and what contribution they make to the final skin structure is often the subject of heated debate. One way of tracking cells on a wound is to use a DNA transfer process to label the cell (Bevan et al., 1997). Retroviral vectors are particularly suited as cell fate markers as they integrate into the target cells genome.

Specialised packaging cell lines are used to generate infectious virus particles carrying a gene of special interest, such as LacZ or GFP (reporter genes). These genes of interest can be used as cell markers to follow the fate of cells in vivo. The packaging cell lines have been generated so that they contain chromosomally integrated expression cassettes for viral gag, pol and env protein, all of which are required to make virus. The gag codes for internal structural proteins, pol for reverse transcriptase and integrase, and env for the viral envelope protein. The env gene determines the specificity of the virus produced, for example, PT67 packaging cells produce amphotropic virus capable of infecting other species of cells.

The packaging cell line is transfected with the plasmid vector DNA (for example by lipofection). The plasmid consists of a gene of interest (such as LacZ or GFP) which has been cloned into proviral DNA, replacing the gag, pol

and env genes. The plasmid vector also contains the packaging signal Ψ which is not present in the packaging cells. On transfecting the plasmid into the packaging cell, DNA is transcribed into the RNA of the virus. The presence of the packaging signal Ψ allows the production of the proteins gag, pol and env by the packaging cell and therefore the production of infectious viral particles containing the gene of interest.

The packaging cell is now a retroviral producing cell which can be used to infect other cells with the gene of interest. On infecting the target cell, the RNA in the virus is reverse transcribed, and the gene of interest is incorporated into the target cell's genome. Only actively replicating cells can be infected. The viral RNA does not include gag, pol or env. Therefore the target cell will continue to express the gene of interest, as will its progeny, but will be incapable of producing further virus.

A selection marker such as the neo gene can be included with the gene of interest. This gene codes for the enzyme neomycin phosphotransferase, which inactivates the aminoglycoside antibiotic neomycin and its analogue G418 which is toxic to mammalian cells. Therefore G418 will be toxic to fibroblasts or keratinocytes which do not express the neo phenotype. Combining the gene of interest with this gene therefore allows the use of selective media containing G418 to promote the survival of packaging cells or target cells expressing the gene of interest. As retroviral integration into a critical gene could be tumorogenic, the non-lifesaving use of such gene markers is typically confined to animal models.

1.13 The pig as a model for wound healing

The gold standard for wound closure in burn surgery is an autologous split skin graft. We need to compare emerging skin replacement technology with this gold standard. Skin replacements have been produced for many years. They have been developed with different combinations of synthetic, dermal and epidermal components. Tissue engineering is beginning to generate increasing numbers of different materials that in combination with different types of cells has the potential to give rise to large numbers of skin replacements. New treatments require scientific testing before use in clinical situations. They will need to proceed through phases of testing. Firstly in the laboratory, often to be followed by animal experiments, and finally introduction into a clinical trial. Clinical trials must gain ethical approval, and for this there should be some evidence from *in vitro* or animal experiments that the material in question will provide a better outcome than the present gold standard.

A wound healing model is judged on its ability to predict how a treatment will behave in humans. Pig skin is anatomically similar to human skin. The epidermis is between 40-120μm in humans and 30-140μm in pigs, both with a dermal epidermal thickness ratio of around 10 to 1 (Vardaxis et al., 1997). The rete-ridges and the subdermal fat thickness are also similar in pigs and humans (Montagna and Yun, 1964). The biochemical structure of porcine dermal collagen is very similar to that of humans, though pigs contain slightly less elastin in their dermis (Marcarian and Calhoun, 1966). The adnexal structures, including hair follicles, are similar in structure in both man and pigs (Meyer et

al., 1978). They both have sparse hair distribution, which is important considering re-epithelialisation occurs from these structures. Immunohistochemical staining has also shown similar patterns of cytokeratin distribution in the epidermis, and similar basement membrane protein deposition in pigs and humans (Wollina et al., 1991).

Small mammals such as the rabbit, guinea pig, rat and mouse are often used due to convenience in housing, care and cost. However, these mammals differ from pigs and humans in that they have a dense layer of body hair, a much thinner epidermis and dermis, and heal mainly from contraction rather than re-epithelialisation. More importantly, when the results of wound healing studies performed in pigs and small mammals were compared to the results of identical studies performed in humans the concordance was found to be 78% between pigs and humans, compared to 53% between small mammals and humans (Sullivan et al., 2001). No wound healing model will completely replicate clinical human wound healing, however, the pig appears to be the best animal model available at present.

1.14 Hypotheses for investigation

The discussion above outlines the latest biotechnological advances that have been made in defining the properties of materials that could serve as skin replacements for permanent wound closure. The ultimate goal is for a one step surgical procedure, which produces an artificial skin identical to native skin.

If commercial collagen matrices are to be used as a one step procedure to resurface burn wounds, the easiest way to achieve this would be to seed the matrix with keratinocytes. The following hypotheses were formulated to further investigate this concept.

- **Cultured autologous keratinocytes when seeded into Integra® artificial skin will produce an epidermis, with migration of keratinocytes through the matrix towards the wound surface.**

- **Non-cultured autologous keratinocytes when seeded into Integra® artificial skin will produce an epidermis, with migration of keratinocytes through the matrix towards the wound surface.**

Fibrin glue has been shown to be a suitable delivery vehicle for keratinocytes. It also has been demonstrated to improve keratinocyte migration. Therefore, it may improve the efficiency of epidermal formation in the above hypotheses. To investigate this the following hypotheses were formulated.

- **Fibrin glue will improve both the efficiency and efficacy of the delivery of subconfluent cultured autologous keratinocytes into the tissue engineered dermal replacement Integra®.**

- **Fibrin glue will improve the efficiency and efficacy of subconfluent cultured autologous keratinocytes delivered to a wound bed in a spray-on-suspension.**

Fibrin glue has been shown to improve the take rate of split skin grafts. It has been shown to have a heamostatic effect and may have an anti-bacterial action. The following hypothesis was formulated to investigate whether a similar benefit could be shown when using fibrin glue with Integra® artificial skin.

- **Fibrin glue will improve the take rate of Integra® artificial skin when used as a keratinocyte delivery system.**

Fibroblasts may improve epidermal formation when used in a composite material. Few studies have shown that the fibroblasts used in these composites survive *in vitro*. The following hypotheses were formulated to further investigate the survival of seeded fibroblasts.

- **Labelled autologous fibroblasts seeded into Integra® artificial skin will contribute to the fibroblast population in the regenerating dermis.**

- **Labelled allogenic fibroblasts seeded into Integra® artificial skin will contribute to the fibroblast population in the regenerating dermis.**

Wounds will contract, even if they are closed with a skin graft. Wounds closed with a split skin graft will contract more than wounds closed with a full thickness skin graft. The rate of wound contraction of wounds closed with artificial skin substitutes has not been described. The following hypothesis was formulated to further investigate this.

- **A wound grafted with Integra® artificial skin and covered with an ultra-thin split skin graft will contract less than an open wound or a wound grafted with a split skin graft alone.**

1.15 Experimental aims

- To evaluate quantitatively the migration of cultured autologous keratinocytes through the collagen glycosaminoglycan matrix.

- To compare the rate of keratinocyte migration using cultured autologous keratinocytes suspended in either culture medium or fibrin glue seeded into the collagen glycosaminoglycan matrix.

- To compare the rate of keratinocyte migration using cultured versus non-cultured autologous keratinocytes seeded into the collagen glycosaminoglycan matrix.

- To assess the percentage of epithelial formation when cultured autologous keratinocyte cell suspensions in culture medium are sprayed onto full thickness wounds, and to compare this with the percentage of epithelial formation when cultured autologous keratinocyte cell suspensions in fibrin glue are sprayed onto full thickness wounds.

- To assess the survival of labelled allogenic and autologous keratinocytes seeded into Integra®.

- To assess the relative wound contraction rates for open wounds, wounds grafted with Integra® artificial skin, and wounds grafted with a split skin graft.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Culture

GIBCO BRL, Life Technologies, Paisley, Scotland.
Dulbecco's Modified Eagle's Media (DMEM) + glutamax +1000mg/l D-glucose
Dulbecco's Modified Eagle's Media (DMEM) + glutamax + 4500mg/l D-glucose.
Opti-MEM® 1 (calcium-free) reduced serum media with GlutaMAX™.
Hanks Balanced Salt Solution (HBSS)
Gentamicin 50 mg ml^{-1}.
Penicillin/ Streptomycin 5000 units ml^{-1}/5000 μg ml^{-1}.
Amphotericin B 250 μg ml^{-1}.
Calcium chloride (0.1M).
Trypsin 0.05%/EDTA 0.02%
Nunc® petri dishes (92mm x 17mm)
Geneticin (G418 Sulphate)
Lipofectamine Plus™

Sigma-Aldrich Co Ltd, Poole, Dorset, England
Rat tail collagen (type I)

Imperial Laboratories Ltd, West Portway, Andover, Hant's, England
Foetal Bovine Serum (Foetal Calf Serum)

Boehringer Mannheim, Lewes, Sussex, England.
Dispase®.

Difco Laboratories, Detroit, Michigan, USA.
Trypsin (0.25%)

Greiner Labortechnik Ltd, Dursley, Gloucestershire, England.
75 cm^2 canted neck filter cap tissue culture flasks
30 ml sterile universal plastic tubes

Marathon Laboratory Supplies, Park Royal, London. England
Falcon® 25 cm^2 tissue culture flasks
Serological pipettes

BD Biosciences Clontech, Oxford, United Kingdom.
RetroPack PT67 packaging cell line

Stratagene, La Jolla, California, USA
The ViraPortTM retroviral reporter vector pFB-hrGFP

2.1.2 Anaesthesia and theatre consumables

Parke-Davies Veterinary Ltd, Pontypool, Gwent, Wales
Ketamine (Vetalar®)

Bayer UK Ltd, St. Edmunds, Suffolk, England
Xylazine (Rompun®)

Zeneca Ltd, Macclesfield, Cheshire, England.
Halothane (Fluothane®)

BOC Ltd, Guildford, Surrey, England.
Nitrous oxide and oxygen

Biorex Laboratories Ltd, London, England
Lignocaine gel (2%)

Smith Kline Beecham Animal Health, Surrey, England.
Amoxycillin/Clavulanic acid for injection (Synulox®).
Amfipen LA, (long acting ampicillin.)

Reckitt & Colman Ltd, Hull, England.
Buprenorphine (Temgesic®)

C-Vet Veterinary Products, Grampian Pharmaceuticals, Lancashire, England.
Carprofen (Xenecarp™)

Civa Ltd, Watford, Hertfordshire, England.
Pentobarbitone (Expirol®)

DePuy Healthcare Ltd, Leeds, England
Chlorhexidine (4%) scrub (Hydrex®)
Chlorhexidine 0.5% in alcohol

Steripak Ltd, Runcorn, Cheshire, England
Normal saline (sodium chloride Ph. Eur. 0.9%w/v)

Seton Healthcare, Oldham, Lancashire, England.
10% povidine iodine in acqueous solution (Betadine®)

Northwick Park Hospital Pharmacy, Harrow England.
Silver Nitrate (0.25% and 0.5% in acqueous solution).
Liquid paraffin

Swann-Morton®, Sheffield, England.
Scalpel blades

Smith and Nephew, Chessington, Surrey, England
Elastoplast® adhesive roll bandages.
Paraffin gauze dressings (Jelonet®).

Johnson & Johnson, Ascot, Berkshire, England.
N/A Ultra®, silicone-coated non-adherent knitted viscose dressings.
Velband®orthopaedic wool bandage

Ethicon Ltd, Edinburgh, Scotland
2/0 and 4/0 silk sutures, 4/0 vicryl and 5/0 mono-filament prolene synthetic sutures

Zimmer, Dover, Ohio, USA.
Zimmer® Air Dermatome

Alan G. Smith Ltd, Gravesend, Kent, England.
'Mitutoyo' dial thickness gauge

Integra Life Sciences Corporation, Plainsboro, New Jersey, USA.
Integra™ Artificial Skin
Baxter Healthcare Ltd, Compton, Berkshire, England.
Tisseel two component fibrin glue.

The Bioengineering Department, Northwick Park Institute for Medical Research, Harrow, England
Percutaneous polytetrafluoroethylene (PTFE) wound isolation chambers

Promedics Ltd, Blackburn, Lancashire, England
Protective pig jackets fashioned from thermoplastic Spectrum® secured with Velcro® straps

Southern Foam, Crawley, Sussex England.
Foam for protective pig jackets

2.1.3 Histology

BDH laboratory supplies, Poole, Dorset, England
Formaldehyde.
Paraformaldehyde.
Glutaraldehyde.
Sodium azide.
Haemotoxylin and Eosin
DPX mountant
Aquamount aqueous mountant

The Department of Histopathology, Queen Victoria Hospital, East Grinstead, England.
Trypan blue.

Tissue-Tek, Miles Inc, Elkhart, IN, USA.
OCT compound, embedding medium for frozen tissue specimens.

GIBCO BRL, Life Technologies, Paisley, Scotland.
5-bromo-4-chloro-3-indolyl-beta-D-galactoside (X-Gal), substrate for the *lacZ* gene product: β-galactosidase.

FSA, Loughborough, England.
Magnesium chloride

Sigma-Aldrich Co Ltd, Poole, Dorset, England.
Potassium ferricyanide.
Potassium ferrocyanide

Professor Irene Leigh, The Royal London Hospital, England.
L1001 anti-keratin 14 - mouse anti-human monoclonal antibody.
LHP2 anti-keratin 10 - mouse anti-human monoclonal antibody.
LH7.2 anti-collagen 7 - mouse anti-human monoclonal antibody.

TCS Ltd, London, England

Goat anti-rabbit FITC conjugate 2° anti-body.

ICN Biomedicals Inc, Costa Mesa, California, USA.

Sheep anti-mouse FITC conjugate 2° anti-body.

Molecular Probes, Oregon, USA.

Propidium iodide (1mg/ml) fluorescent nuclear stain

BDH laboratory supplies, Poole, Dorset, England.

Aquamount® improved anti fade mountant

2.2 METHODS

2.2.1 Culture and transport media

3T3 growth medium

Product	**Concentration**
DMEM with GlutaMAX™ 1000mg/l D-glucose	
Foetal calf serum	10%
Penicillin/Streptomycin	100 units ml^{-1}/100 $\mu g\ ml^{-1}$

Transport medium

Product	**Concentration**
Opti MEM 1 with GlutaMAX™	
Foetal calf serum	1%
Penicillin/Streptomycin	600 units ml^{-1}/600 $\mu g\ ml^{-1}$
Amphotericin	1.25 $\mu g\ ml^{-1}$

Pig keratinocyte growth medium

Product	**Concentration**
Opti MEM 1 with GlutaMAX™	
Foetal calf serum	1%
Penicillin/Streptomycin	100 units ml^{-1}/100 μg ml^{-1}
Final $[Ca^{2+}]$	0.5 mM

PT67 growth medium

Product	**Concentration**
DMEM with GlutaMAX™ 4500mg/l D-glucose	
Foetal calf serum	10%
Penicillin/Streptomycin	100 units ml^{-1}/100 μg ml^{-1}

2.2.2 Cell lines

All tissue culture procedures were performed in a Jouan Class II tissue culture hood. Cell cultures were grown in an LEEC incubator maintained at 37°C, 98% relative humidity, and 5% CO_2.

Swiss 3T3 fibroblasts were cultured in 75cm^2 flasks using 10mls of 3T3 growth medium. When flasks reached 75% confluence the medium was removed and the cells washed in 10mls of HBBS solution. This was again removed and the cells were dispersed using 2.5mls 0.05% trypsin / 0.02% EDTA incubated for 5 mins at 37°C. The trypsin was then neutralised with 2.5mls of 3T3 growth medium. The cells were centrifuged at 1000rpm for 5 minutes and the supernatant discarded. The cells were then re-suspended in 5mls of 3T3 growth medium and a cell count performed using a haemocytometer. The cells were either re-seeded in a ratio 1:10 into 75cm^2 flasks for continuation of the cell line, or were irradiated for use with pig keratinocyte culture.

The retroviral producer cell line PT67lacZnls + neo clone 6 and PT67GFP cell lines were maintained in the same way as the Swiss 3T3 fibroblasts, though PT67 growth medium was used.

2.2.3 Irradiation of 3T3 cells

Pig keratinocytes will grow in culture if supported by a layer of fibroblasts producing various growth factors. If the fibroblasts are not pre-irradiated they

will thrive in the culture medium, replicating at a faster rate than the keratinocytes and exhausting the nutrients supplied to them by the medium. If the fibroblasts are given a sub-lethal dose of irradiation they will continue to produce growth factors but will be unable to replicate. The optimal dose has been calculated as 60 Grays (1 Gray = 100 Rads). The radiation source used for this study was capable of delivering 100,000 rads in August 1997.

To calculate the present gammacell activity:

Original activity August 1979100,000 R/Hr

Decay ratio from isotope table

t = real time

T = isotope half life

t/ T = Date / half life = (8/2000-8/1979) /30.2

= 21/30.2

= 0.6954

Best value fit from decay table lies between 0.624 and 0.6156 roughly 0.6199

Original activity = 100,000 R/Hr

Present activity = 100,000x0.6199 = 61,990 R/Hr

1,033 R/min

3T3 cells require 6,000 rads, therefore they required irradiation at present activity for 5.8 mins. Therefore, when required for pig keratinocyte culture, 3T3 cells or PT67 cells were exposed to the ^{137}Cs source for 5.8 minutes prior to plating onto the collagen coated flasks.

2.2.4 Collagen coating of culture flasks

Pig Keratinocytes will grow optimally in vitro on culture flasks coated with rat tail type 1 collagen (Hengge et al., 1996). 75cm^2 tissue culture flasks are coated by contact with 10mls of a collagen solution for 2 hours. The solution is made by dissolving 10mg of rat tail collagen type 1in 40mls of 0.1M acetic acid and leaving on a stirrer for 2 hours. This is then made up to 200mls by the addition of 160mls of tissue culture grade distilled water. Chloroform is then added to sterilise the solution by dripping the liquid down a glass rod placed at the bottom of the container, and this is left overnight at 4°C. The sterile solution containing the rat tail type 1 collagen is then pipetted from the underlying chloroform.

Once the flasks were coated and had been allowed to dry with the caps off, they could be stored at room temperature for up to one month. Immediately prior to use they were rinsed with 10mls of Hanks Balanced Salt Solution (HBSS).

2.2.5 Pig Keratinocyte Isolation

Under general anaesthetic skin biopsies were taken from the para-vertebral region of the pigs using an air dermatome. Split thickness skin of around 200-300μm thick measuring 5x4 cm was harvested under sterile conditions and immediately placed into transport medium. The skin biopsies were then washed five times in HBSS at 37°C containing 1.25μg ml^{-1} of amphotericin, using fresh solution for each rinse. Each biopsy was divided into thin strips 2mm wide, still attached at one end, and the epidermis was lightly scored with a scalpel. The

skin was then incubated at 37°C in 0.25% trypsin in HBSS containing 2.5μg/ml of amphotericin for 3 hours. The scoring allowed the trypsin to separate the epidermis from the underlying dermis, such that the epidermis could be gently teased off with a scalpel blade. The epidermal paste was then further diced and re-suspended in the HBBS 0.25% trypsin solution for 10 minutes. The suspension was then filtered through a cell sieve (250 μm mesh) to remove any remaining debris. The cell suspension was then centrifuged at 1000 rpm for 5 minutes and the supernatant discarded. The cells were then re-suspended in complete growth medium containing 10% foetal calf serum in order to neutralise the trypsin. The suspension was once again centrifuged and the supernatant again discarded. The cells were finally re-suspended in 10 mls of keratinocyte growth medium, and a cell count performed using a haemocytometer. A biopsy measuring 5x4 cm would produce about $16x10^6$ keratinocytes with about 60% cell population viability, as measured using tryphan blue uptake.

2.2.6 Pig keratinocyte culture

The isolated pig keratinocytes were then seeded onto collagen coated flasks ($75cm^2$) which had been pre-seeded with $2x10^6$ irradiated 3T3 cells. The 3T3 cells were irradiated with 60 Gray from a ^{137}Cs source and plated onto the flasks in pig keratinocyte culture medium 2 hours prior to the pig keratinocyte isolation. A seeding density of $4x10^6$ per $75cm^2$ flask was used for the keratinocytes, which produced a 90% confluent flask in 5-6 days. The keratinocyte medium was changed every third day.

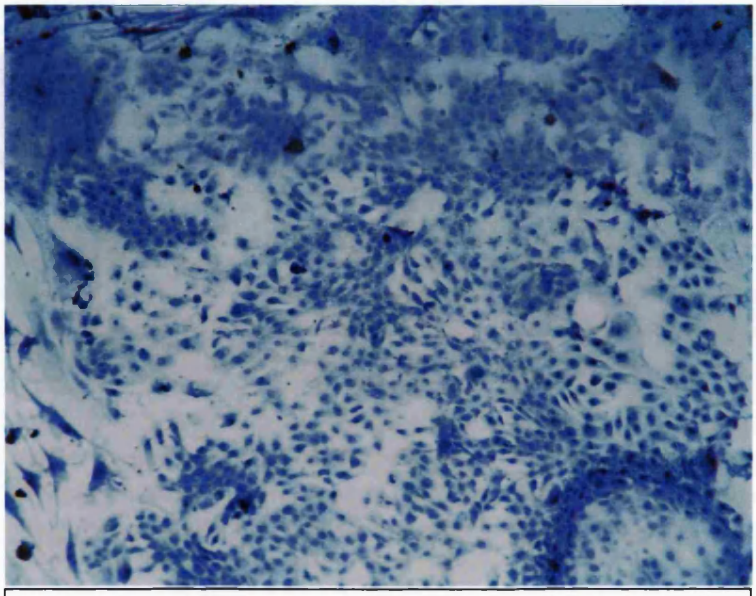


Figure 2.1 – Cultured keratinocytes.
X 20 view of a culture flask stained with Haematoxylin. This flask contains irradiated 3T3 cells and pig keratinocytes. The keratinocytes in the flask are 98% confluent.

When 90% confluent (Figure 2.1) the flasks were passaged by dispersing the keratinocytes with 0.05% trypsin / 0.02% EDTA. These were then subcultured in a ratio of 1:3 onto collagen coated flasks pre-treated with irradiated 3T3 cells. These secondary cultures reached 90% confluence in 2-3 days.

2.2.7 Retroviral transduction of pig keratinocytes with vectors expressing the green fluorescent protein gene

Second passage autologous keratinocytes were isolated from the animals as above. These were seeded into 75cm^2 flasks in a density of 4x10^6 per flask with 10ml of retrovirus containing keratinocyte culture medium. The keratinocyte medium was prepared in advance by incubation at 32°C for 72 hours with the

retroviral producer line PT67pFB-hrGFP A2 clone (without G418). The keratinocytes were incubated with the retroviral containing culture medium for 18 hours at 32°C. They were then rinsed and returned to fresh keratinocyte culture medium.

2.2.8 Calculation of transduction frequency

Flasks containing transduced keratinocytes were examined under UV light. The keratinocytes expressing Green Fluorescent protein were counted in ten fields at x40 magnification. This was compared with the total number of keratinocytes in ten fields at the same magnification to give a percentage transduction rate.

2.2.9 Pig fibroblast isolation

Under general anaesthetic skin biopsies were taken from the para-vertebral region of the pigs using an air dermatome. Split thickness skin of around 200-300μm thick measuring 5x4 cm was harvested under sterile conditions and immediately placed into transport medium. The skin biopsies were then washed five times in warm HBSS containing 1.25μg ml^{-1} of amphotericin, using fresh solution for each rinse. The epidermis was removed by incubation at 37°C in 0.25% trypsin in HBSS containing 2.5μg ml^{-1} of amphotericin for 20 hours at 4^0C. The epidermis was discarded and the dermis washed in warm HBSS, and then in 3T3 culture medium to neutralise any trypsin remaining in solution. The dermis was cut into small pieces and then incubated in 0.1% collagenase A in DMEM 1g/l glucose (30mg / 10ml) for 4 hours at 37°C. The suspension was filtered through a cell sieve (70 μm mesh) to remove any remaining debris. The

cell suspension was then centrifuged at 1000 rpm for 5 minutes and the supernatant discarded. The resulting pellet containing pig fibroblasts was then suspended in 3T3 growth medium.

2.2.10 Retroviral transduction of pig fibroblasts with vectors expressing the *lacZ nls* gene

Porcine fibroblast cultures were isolated as above and grown in 3T3 growth medium using previously described methods(Ghosh et al., 1997). Retroviral transduction was carried out at 32°C as described by Kotani et al 1994 (Kotani et al., 1994) using *MFGlacZnls* (Ferry et al., 1991) from a producer line constructed by transfection of a PT67 cell line (Miller and Chen, 1996). Porcine fibroblasts were isolated from split thickness skin grafts and cultured for 6 days prior to being exposed to the *MFGlacZnls* retrovirus over a subsequent period of 5 days. The fibroblasts were labelled with the *lacZnls* reporter gene using supernatant transduction with the *MFGlacZnls* virus produced from a PT67 cell line. Porcine fibroblast cultures were subjected, on 2 separate occasions, to overnight incubation at 32°C with filtered supernatant produced from a culture of the PT67 virus producing cell line that had been incubated at 32°C for 72 hours in an excess volume of growth medium. This virus enriched supernatant was then placed onto the fibroblast cultures. Following overnight incubation at 32°C, the transduced cultures were returned to ordinary fibroblast media and 37°C incubation.

2.2.11 Calculation of transduction frequency

Flasks of transduced fibroblasts were stained for β-galactosidase activity using the X-gal substrate to determine the transduction frequency of the *lacZnls* labelled cultures (Figure 6.1). No positive selection was used for the transduced cells. Counts from 15 representative fields from x-gal stained flasks of labelled fibroblasts were taken to determine the transduction frequency of the *lacZnls* labelled cultures.

2.2.12 Animals

Animal experiments were carried out at Northwick Park Institute for Medical Research, Harrow, London, under the UK Home Office project licence PPL70/4100 and personal licence PIL80/7654. Outbred female Large White pigs from a single supplier were used for the animal experiments.

Six-week old pigs were introduced to their new environment one week prior to the start of any procedures in order to allow acclimatisation to their new surroundings and reduction of stress. Pigs were kept in individual pens but in a room with other pigs to allow social interaction using standardised animal husbandry techniques. Standard delousing prophylaxis was administered as a one-off dose of 1ml sub-cutaneous Ivomectin.

2.2.13 Anaesthesia

Animals were sedated with an intramuscular injection of xylazine 1 $mgKg^{-1}$ and ketamine 5 $mgKg^{-1}$. The pigs were then transported to the operating theatre **(Figure 2.2)** and anaesthesia was induced with halothane 2-5% (Fluothane®) mixed with 3-5 L min^{-1} of nitrous oxide/oxygen (50:50) via a face-mask. Pressure point care was observed throughout using padding.

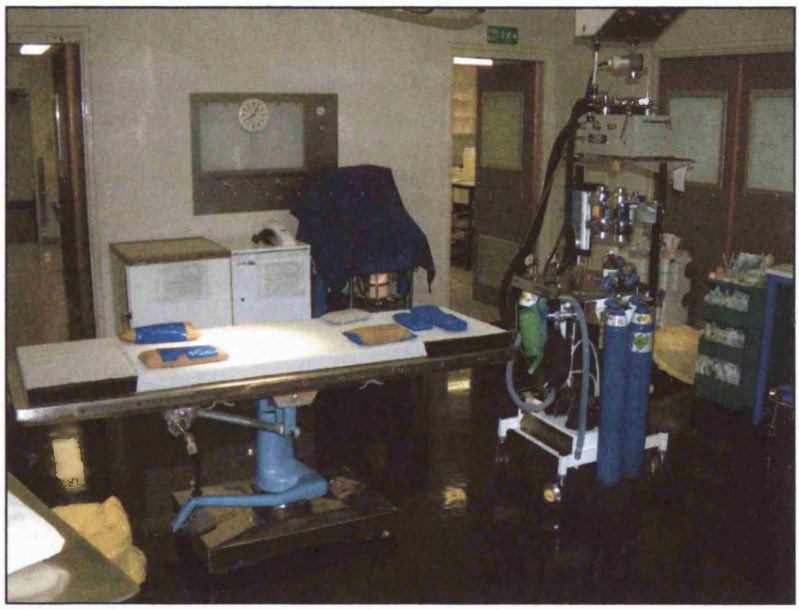


Figure 2.2 – Operating theatre for pig experiments.

A single dose of antibiotic prophylaxis was used for all procedures including dressing changes unless otherwise specified. 350 mg amoxycillin and 87.5 mg clavulanic acid (Synulox®) was given by intramuscular injection on induction. Buprenorphine (Temgesic®) 4 μg kg^{-1} or carprofen (Xenecarp™) 4 mg kg^{-1} were used for post-operative analgesia as a single subcutaneous injection. If a

split skin graft was harvested this was additionally dressed with topical 2% lignocaine gel.

Euthanasia was carried out after the final procedure without reversing it. An intra-muscular injection of 140 mg kg^{-1} of pentobarbitone (Expirol®) was administered.

2.2.14 Harvesting of Split Thickness Skin Grafts

After shaving the designated graft site the animal was washed with a scrubbing brush in 4% aqueous chlorhexidine. A surgical prep of 0.5% chlorhexidine in alcohol was then applied and the animal was draped with sterile surgical towels. Split thickness skin grafts were harvested from the para-vertebral region of the pig using a Zimmer air dermatome set at 8-10. In order to isolate sufficient keratinocytes a split skin graft measuring 5 x 5 cm was harvested. This was then placed in a sterile flask of transport medium and the wound was dressed with topical lignocaine gel, paraffin gauze dressing (Jelonet®) and saline soaked surgical gauze.

2.2.15 Creation of full thickness PTFE chambered wounds

Six full thickness wounds were made on each pig, three on either side (**Figure 2.3**). After shaving the designated area each animal was washed with a scrubbing brush in 4% aqueous chlorhexidine. A surgical prep of 0.5%

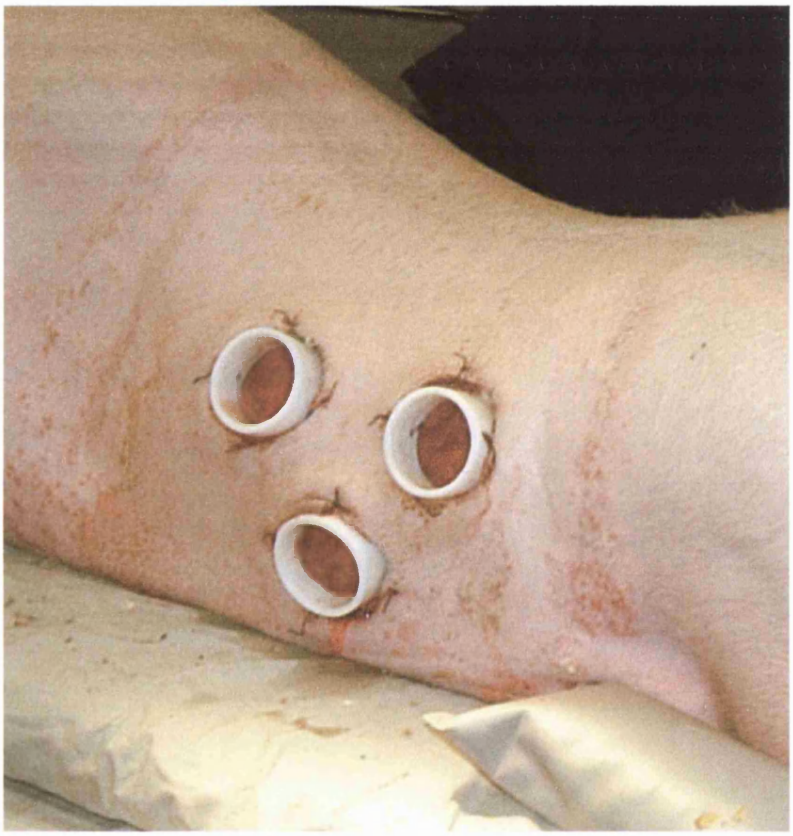


Figure 2.3 - PTFE chambers in position on pig flank.
The chambers are 4 cm in diameter and prevent the wound from contracting, as well as preventing re-epithelialisation from the wound margins. They are held in position with four silk sutures.

chlorhexidine in alcohol was then applied and the animal was draped with sterile surgical towels. Full thickness wounds were measured using a template 4 cm in diameter, and the wounds made using a scalpel. Three circular wounds

were created on each flank. The wounds were made through the epidermis, dermis, subcutaneous fat and the panniculus carnosus layer, such that the fascia over the skeletal muscle acted as the wound bed. The edges of the wound were then undermined for about 1 cm to allow the flanges of the PTFE chambers to be inserted under the skin. The PTFE chambers prevent ingrowth of the epidermis and reduce wound contraction. A 1cm vertical incision was made at the superior border of the wound to allow insertion of the chamber. The experimental wound graft or treatment was then secured as per protocol below and the chambers inserted over the grafts. The chambers were secured with four 2/0 silk sutures. The wounds were dressed with lightly packed surgical gauze and the chambers covered with Velband® orthopaedic wool secured with Mefix® and Elastoplast® adhesive tape. Custom made rigid jackets of thermoplastic Spectrum® were applied and secured in place with Velcro® straps **(Figure 2.4).**

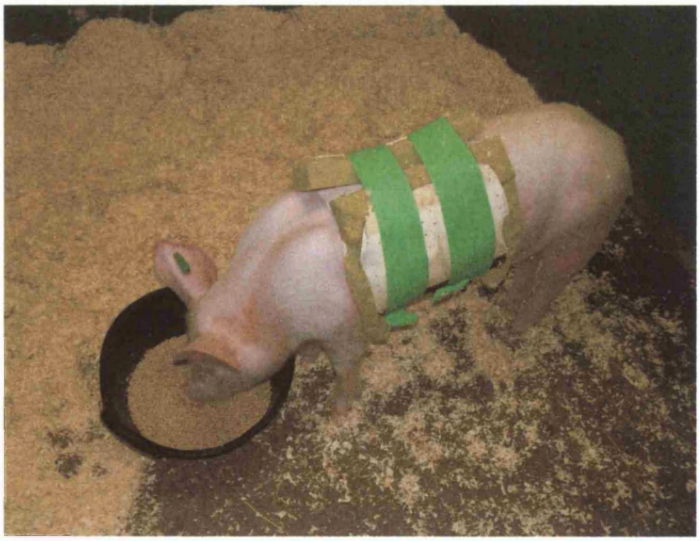


Figure 2.4 – Custom made protective jacket
The pigs were kept in separate pens and wore custom made protective jackets to prevent them from interfering with the wounds. Dressings were changed twice weekly.

To reduce operative time two surgeons created the wounds, one working on each flank. The wounds were distributed on the pigs with similar graft types applied by both surgeons in equal numbers. This was to eliminate any operative bias.

2.2.16 Wound Chamber Grafts

2.2.16.1. Integra®

Integra® is supplied in isopropyl alcohol and therefore requires rinsing twice in two litres of normal saline. Once this was completed the Integra® was cut into circles using the wound chambers as a template. After the wound had been cut into the flank, but before insertion of the chamber, the circle of Integra® was sutured to the base of the wound with a continuous circumferential 4.0 Prolene® suture. The siliconised surface of the Integra® was placed outermost.

2.2.16.2. Integra® and fibrin glue

The Integra® was treated as above, but prior to suture a mixture of 0.5ml of the fibrinogen/aprotinin component of the fibrin glue mixed with 0.5ml of keratinocyte growth medium was applied to the under-surface of the Integra® using a needle-less syringe. The thrombin component of the fibrin glue was applied directly to the wound bed with a needle-less syringe just prior to the Integra®.

2.2.16.3. Integra® and suspension of keratinocytes

Keratinocytes were delivered into Integra® by dripping a suspension of cells onto the non-siliconised surface **(Figure 2.5).** The cell concentration used was 6.25 x 10^6 per ml and 1 ml of solution was applied to each wound. This delivered a keratinocyte load of 5 x 10^5 keratinocytes per cm^2 to the Integra® in

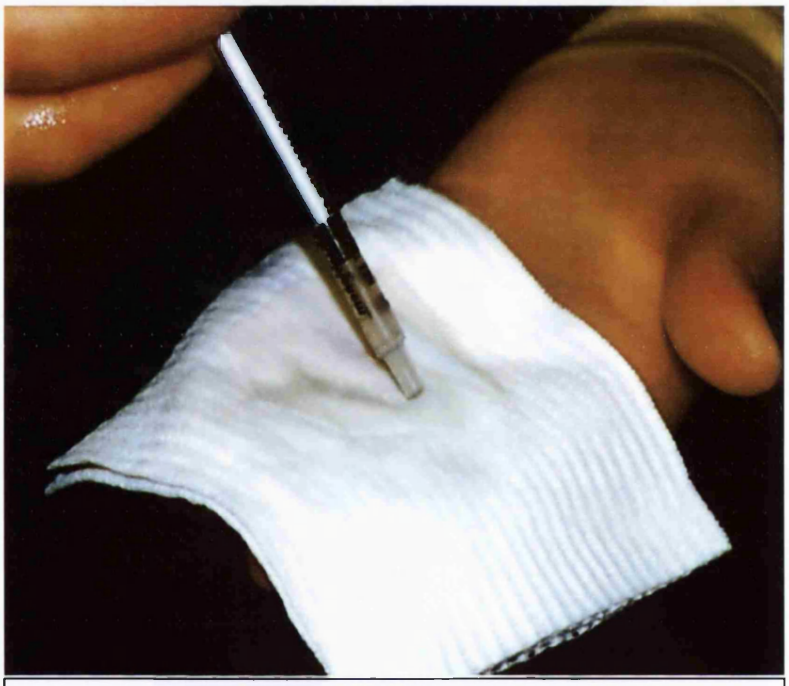


Figure 2.5 – Seeding Integra® with suspension of cultured autologous keratinocytes.
The suspension of keratinocytes was dripped evenly onto the Integra® after it had been patted dry with a dry gauze swab. Histological analysis demonstrated that the cells were evenly distributed throughout the matrix.

a volume of fluid that was sufficient to soak the Integra® without flooding it. This cell density was chosen based on previous studies by Compton *et al* (Compton et al., 1998), which demonstrated a confluent epithelium at two

weeks when centrifuging a collagen-glycosaminoglycan matrix with 5 x 10^5 keratinocytes per cm^2.

2.2.16.4. Integra® and keratinocytes in Fibrin Glue

The cells were suspended in the fibrinogen/aprotinin component of the glue. Tisseel fibrin glue (Baxter, UK) was used, which is a commercial product made from pooled human fibrinogen, available as 1, 3 and 5 ml packs. The reconstituted powder contains 100 - 130 mg total protein per ml of which 75 - 115 mg is fibrinogen. Keratinocyte medium containing 6.25 x 10^6 cells was centrifuged at 1000rpm for 5 minutes and the supernatant removed. The cells were reconstituted with 0.5 ml of keratinocyte growth medium and 0.5 ml of the fibrinogen/aprotinin solution. This delivered 6.25 x 10^6 keratinocytes and 30mg of fibrinogen to each wound. Thrombin was applied directly to each of the wound beds as a dose of 250IU. This concentration of fibrinogen was used based on data from studies using fibrin glue cell suspensions with cadaveric allograft (Stark and Kaiser, 1994;Horch et al., 1998). One ml of the glue was sufficient to attach 100 cm^2 area of allograft skin. A slightly higher fibrinogen concentration was used for this study as Integra® is extremely absorbent.

2.2.16.5. Integra® and suspension of fibroblasts

A seeding density of $5x10^5$ per cm^2 was used. Each wound measured 12.5 cm^2 so a total of $6.25x10^6$ fibroblasts were applied to each wound. This was applied to the non-siliconised surface of the Integra® after it had been washed three

times in saline and then patted dry with a gauze swab. The cells were delivered to the Integra® in 2 mls of fibroblast culture medium.

2.2.17 Wound chamber cell sprays

Some wounds were treated with keratinocyte suspensions delivered to the wound bed in aerosols. Pre-confluent autologous keratinocytes (third passage) were suspended in a concentration of $1x10^6$ cells per ml of pig keratinocyte culture medium. These were sprayed onto the wound bed in 2ml aliquotes. Alternatively pre-confluent autologous keratinocytes (third passage) were suspended in a concentration of $1x10^6$ cells per ml in a mixture of 1ml of pig keratinocyte culture medium mixed with 1 ml of the fibrin/aprotinin component of Tisseel fibrin glue (Baxter), mixed as per manufacturers instructions. The suspensions were sprayed at a distance of 10 cm from the wounds. In the latter group the thrombin component of the fibrin glue kit (500iu in 1 ml) was applied to the wound bed immediately prior to spraying. The device used for spraying the suspensions consisted of a 5 ml syringe with a spray cap supplied by Coster Aerosols Ltd, Stevenage, Herts, UK (cat no: V06222). This was sterilised by steam autoclaving for 20 minutes at 121^0C.

2.2.18 Histology

Whole wound excision biopsies were performed at fixed time periods throughout the study. The dissection was performed to include the most superficial part of the underlying muscle. The chamber was removed and the residual wound was dressed with saline soaked gauze. The excision biopsy was

then cut into quadrants and the four faces marked were prepared for histology by snap freezing in O.C.T. using liquid nitrogen. The biopsies were placed into a silver foil cube with the relevant foil face marked. They could then be stored at -40°C.

2.2.19 Cryosectioning

15 μm thick sections were cut using a rocking microtome mounted inside a refrigerated insulation cabinet.

2.2.20 Haematoxylin and Eosin (H&E)

Slides were air-dried. They were then immersed in haematoxylin for 30 seconds and then rinsed in tap water for five minutes. They were then placed in eosin for a further five minutes. The slides were finally dehydrated through ascending strengths of alcohol (30%,70% and 100%) followed by immersion in Xylene. They were mounted in D.P.X. with a 50mm cover slip.

2.2.21 Immuno-fluorescent histology

The primary antibodies were mouse anti-human monoclonal antibodies which cross react with pig antigens. LHP2 anti-keratin 10, L1001 anti-keratin 14, LH6B anti-keratin 6 and LH7.2 anti-collagen VII mouse anti-human monoclonal anti-bodies were supplied by Professor Irene Leigh, Royal London Hospital, UK. They were obtained as tissue culture supernatants and used without dilution. Slides were fixed for 20 minutes in methanol and acetone (1:1), and were then rinsed in P.B.S. Each slide was incubated with 150μl of the

primary antibody solution for 1 hour. The slides were rinsed twice in P.B.S. and then incubated for 45 minutes with 150µl of the secondary antibody (sheep anti-mouse FITC conjugate) at a dilution of 1 in 100. The slides were rinsed in P.B.S. again, and then 150µl of propidium iodide (1mg/ml) was applied at a dilution of 1 in 50 for 30 seconds. The slides were again rinsed in P.B.S and then mouted in anti-fade mountant. Slides were photographed immediately but could be stored at -20°C for a month.

Each batch of slides included a negative control with no primary antibody, and a positive control of normal pig skin.

2.2.22 X-Gal Staining

Flask specimens or sections on slides were rinsed twice with phosphate buffered saline (PBS) and then fixed for 5 minutes in PBS containing 2% formaldehyde and 0.2% glutaraldehyde at room temperature. After a further rinse in PBS the specimens were incubated for 2 hours at 37°C in X-Gal solution. After rinsing in PBS the slides were mounted in aquamount with a 50mm cover slip.

Table 2.1 X-gal solution constituents.

Constituent	Final Concentration
Magnesium chloride	1mM
Potassium Ferrocyanide	5mM
Potassium Ferricyanide	5mM
X-Gal solution (5% w/v in DMSO	0.05%

CHAPTER 3

THE USE OF FIBRIN GLUE TO SEED INTEGRA® ARTIFICIAL SKIN WITH CULTURED AUTOLOGOUS KERATINOCYTE SUSPENSIONS

3.1 Introduction

Integra® has been used extensively for burn reconstruction since its original description by Yannas *et al* in 1980 (Yannas and Burke, 1980;Yannas et al., 1980). Integra® is applied to a wound bed and becomes bio-integrated to form a "neodermis". The size of the pores between the collagen (70-200μm) is crucial in allowing host cells to migrate through the structure, resulting in the progressive replacement of the bovine collagen with the host's collagen. Conventional use of the Integra® allows bio-integration of the matrix, followed by removal of the silicone layer and application of an ultra-thin split skin graft. However, some authors have attempted to seed the Integra® with autologous keratinocytes in order to avoid the need for skin graft application. Keratinocytes in suspension have been centrifuged into an Integra-like matrix so that they lie at the collagen/silicone interface, this complex was then grafted into a pig wound (Butler et al., 1998;Compton et al., 1998). The silicone acts as an epidermis while the keratinocytes mature and differentiate into an autologous epidermis over the "neodermis". Cultured human keratinocytes have also been shown to produce a surface epithelium when seeded into Integra® (Kremer et al., 2000), incubated *in vitro* and subsequently grafted onto athymic mice. The material exhibited good wound adherence, complete healing, minor wound contraction and had the potential to reconstitute an elastic functional and durable human skin. Similar findings have also been reported in a pig model for non-centrifuged cultured keratinocytes (Jones et al., 2000).

The possible benefit of using fibrin glue to secure Integra® has yet to be reported. It is well accepted that firm adhesion of Integra® is essential to achieve high take rates (Grant, 2001). Fibrin glue has some advantages when used in burn patients to secure skin grafts (Currie et al., 2001). Fibrin glue has been shown to have a haemostatic effect after burn debridement (Ihara et al., 1984), and if used as a topical haemostatic agent to secure skin grafts, resulted in an estimated blood loss of $0.5ml/cm^2$ of skin graft, compared with $0.98ml/cm^2$ in the control group (McGill et al., 1997). Fibrin glue has some advantages when used in burn patients to secure skin grafts (Currie et al., 2001) and it has also been shown to improve graft "take". Vibe et al (Vibe and Pless, 1983) demonstrated an improvement in split skin graft area take from 83% to 92% with the use of fibrin glue in 20 patients. More significantly he noted an improvement in graft take from 44% to 88% for grafts placed at difficult areas, such as over mobile muscle or close to skin folds. This is a relatively constant finding throughout the literature: a slight improvement in simple graft take, but significant improvement in areas that are difficult to graft (Lilius, 1987). Fibrin glue has been used to secure sheets of cultured autologous keratinocytes to wounds in animal models (Xu et al., 1996;Auger et al., 1993) with a 20% improvement in the take rate of the grafts.

3.2 Study objectives

It is our hypothesis that if cultured autologous keratinocytes could be seeded into Integra® using the fibrin component of fibrin glue as the suspension medium, then this may combine the advantages of improved haemostasis and

graft take to these seeded cell matrix composites. The aim of this study was to compare the rate of epithelium formation using Integra® seeded with cultured autologous keratinocytes in standard growth medium, with Integra® seeded with cultured autologous keratinocytes in the fibrin component of a commercially available fibrin glue (Tisseel, Baxter, UK).

3.3 Experimental design

3.3.1. Animals

Five pigs were used for this study, with a total of 30 wounds. Six wounds were made on the flanks of each animal, down to skeletal muscle. The PTFE chambers were inserted as described in Materials and Methods (cf. 2.2.15). This prevented re-epithelialisation from the wound margins and prevented the wounds from contracting.

3.3.2. Wound Distribution

Ten wounds were grafted with Integra® impregnated with keratinocytes suspended in 1 ml of pig keratinocyte medium (cf. 2.2.16.3), 2 wounds on each pig. These were compared to ten wounds grafted with Integra® impregnated with keratinocytes suspended in 0.5 ml of the fibrinogen/aprotinin component of fibrin glue mixed with 0.5 ml of pig keratinocyte medium (cf. 2.2.16.4), 2 wounds per pig. In this group the thrombin component of the fibrin glue was applied to the wound bed immediately prior to grafting. The remaining wounds were used for controls. Five wounds were grafted with Integra® alone, one per

pig, and five wounds were grafted with Integra® impregnated with the fibrin component of fibrin glue, one per pig (Figure 3.1).

3.3.3.Keratinocyte isolation and culture

For each pig a split thickness skin graft was harvested from the flank of the

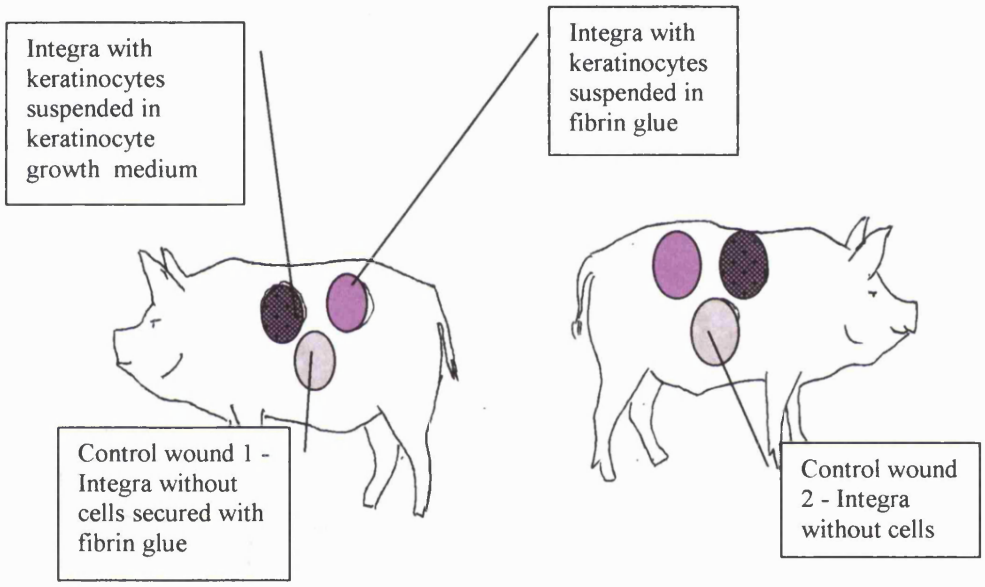


Figure 3.1 – Wound distribution of pigs for study.
The wounds were distributed as shown for pig 1. The allocation was rotated on each side for each of the other four animals. This ensured that in subsequent animals each graft type was placed on different wound positions on the flank. This eliminated any bias due to the wound position.

animal, and the keratinocytes were isolated according to the protocol in Materials and Methods (cf. 2.2.5). The keratinocytes were cultured according to the protocol in Materials and Methods (cf. 2.2.6). The autologous keratinocytes were cultured through four passages over a three week period, and then

harvested from culture whilst still in a pre-confluent state. The autologous keratinocytes were counted with a haemocytometer, then centrifuged and re-suspended in keratinocyte growth medium in a concentration of 6.25 x 10^6 keratinocytes per ml for seeding into the Integra®. The split skin graft donor sites were completely healed in all cases by the time the wound chambers were inserted three weeks later.

3.3.4 Preparation of Wound Chamber Grafts

The porcine model described by Kengesu *et al* was used (Kengesu *et al*, 1993) The Integra® grafts were prepared according to the protocol in Materials and Methods (cf. 2.2.16). Care was taken to ensure the Integra® was thoroughly rinsed to remove the isopropyl alcohol which would be toxic to the cultured keratinocytes. The keratinocytes were delivered into the Integra® in a concentration of 6.25 x 10^6 keratinocytes per ml, which delivered a keratinocyte load of 5 x 10^5 keratinocytes per cm^2 of Integra®. Initially it was proposed to centrifuge the keratinocytes into the Integra® as previous authors have centrifuged keratinocytes into collagen-glycosaminoglycan (CG) matrices at 50G for 15 minutes (Butler et al., 1999). However, a trial run demonstrated that centrifugation at this rate did not improve the penetration of the Integra® by the cultured keratinocytes. This was assessed by histological section, and the keratinocytes penetration towards the silicone layer assessed visually. High rpm producing 800G for an hour also had little effect. This must be due to the effect of the pore size of Integra® compared to the pore size of matrices used by other authors. The best penetration was achieved by patting the Integra® dry with a sterile gauze swab and then dropping the suspension onto the non-siliconised

surface. The suspension was absorbed by the matrix in a similar manner to blotting paper absorbing ink. Histological section demonstrated penetration up to two thirds of the way towards the silicone and this was not improved by subsequent centrifugation. Therefore this simple technique was adopted for seeding the Integra®.

3.3.5 Wound biopsies and Histology

Whole wound excision biopsies were performed at one, two, three, and four weeks. The dissection was performed to include the most superficial part of the underlying muscle. The chamber was removed and the residual wound was dressed with saline soaked gauze. The excision biopsy was then cut into quadrants **(Figure 3.2)** and the four indicated faces were prepared for histology and immunohistochemistry (cf. 2.2.18-21).

3.3.6 Quantification of epithelial cover

The percentage of epithelial cover was calculated by examination of histological sections. Four sections from each wound were examined, representing two cross sections at 90^0 to one another **(Figure 3.2)**. The percentage of mature epithelium was calculated by grid overlay on each slide and the mean percentage of epithelial cover calculated for each wound

3.3.7 Quantification of Integra® "take" rate

Integra® "take" (the assimilation of matrix within the structure of the healing wound) was estimated by examination of histological sections. If Integra® was assessed as present and was seen to be acting as a template for collagen deposition **(Figure 3.3)** the slide was scored as 1. If no Integra®, or Integra® remnants not acting as a template could be seen, the slide was scored as 0. The percentage of Integra® "take" was then estimated for each group. This was done by adding up the score for each wound (a maximum of 4), adding the score for wounds grafted with the same combination of Integra, fibrin and keratinocytes, and then converting this score to a percentage of the maximum possible. This method measures Integra® "take" apparent at the stated biopsy time point, and not at day 10 upon removal of the silicone membrane.

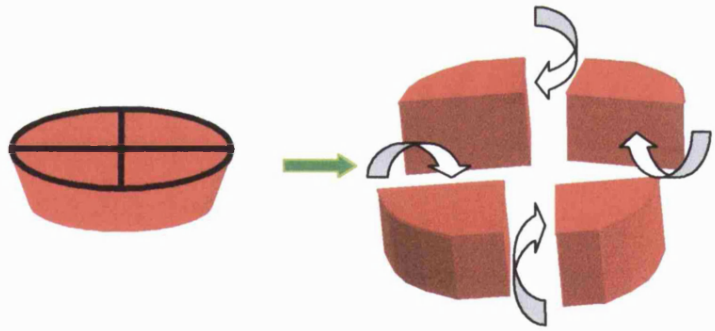


Figure 3.2 – Division of excision wound biopsy into quadrants for histological section. The four histological sections from each wound represent two 90^0 cross sections of each wound.

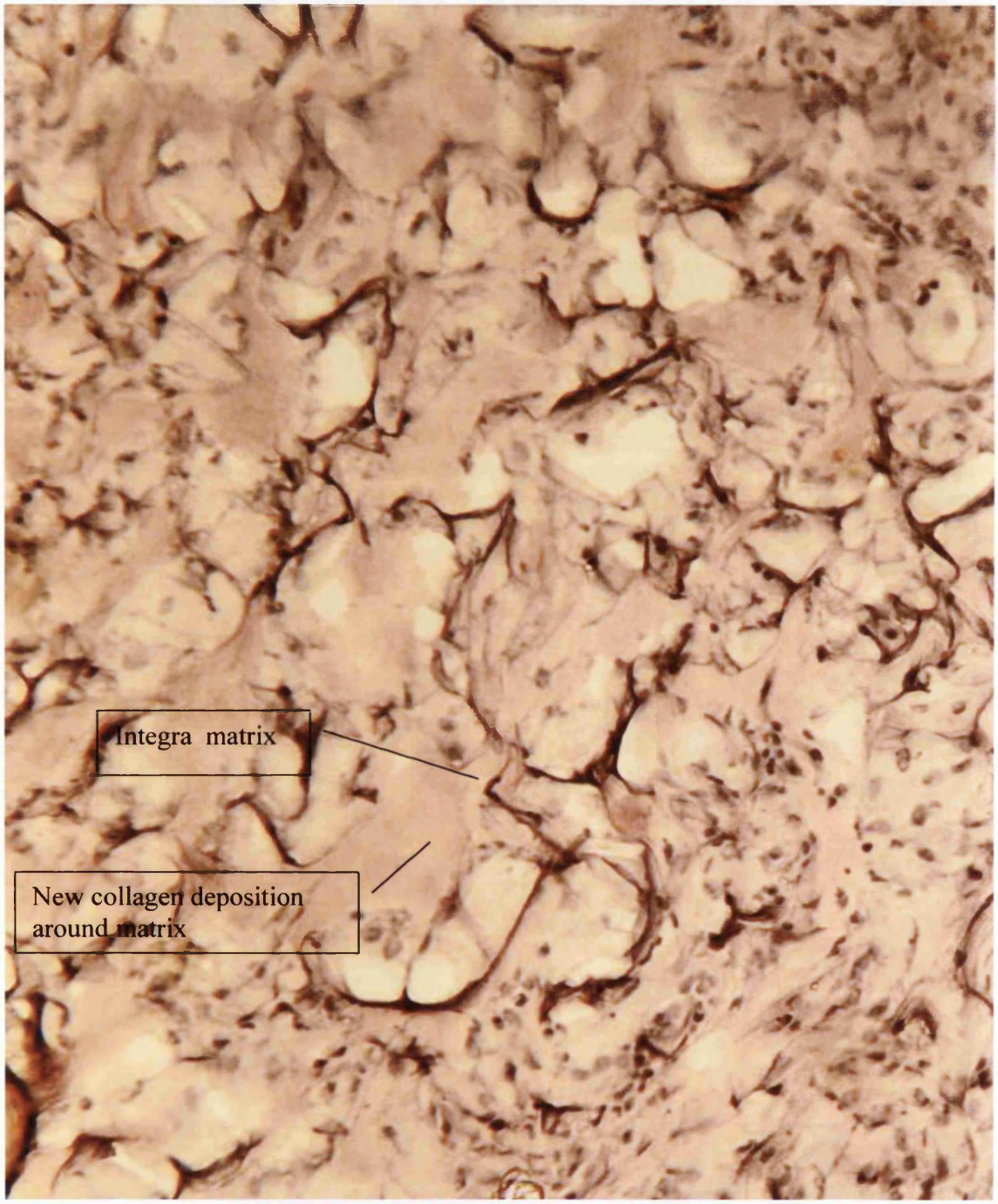


Figure 3.3 - Estimation of Integra take rate (H&E x100)
H&E section showing Integra still visible in the wound biopsy at three weeks. Collagen is beginning to be deposited in an orderly fashion around the matrix. If this picture was seen the quadrant was scored with 1 point. This gave a total score of 4 possible for each wound. From this data the percentage of Integra take was estimated in each group.

3.4 Results

3.4.1 Validation of epithelial calculation

The technique for epithelial calculation (c.f. 3.3.6) was validated by a comparison of two independent observers. The plot of observer 1 versus observer 2 gave a straight line with r = 0.972 **(Figure 3.4)**. The percentage of

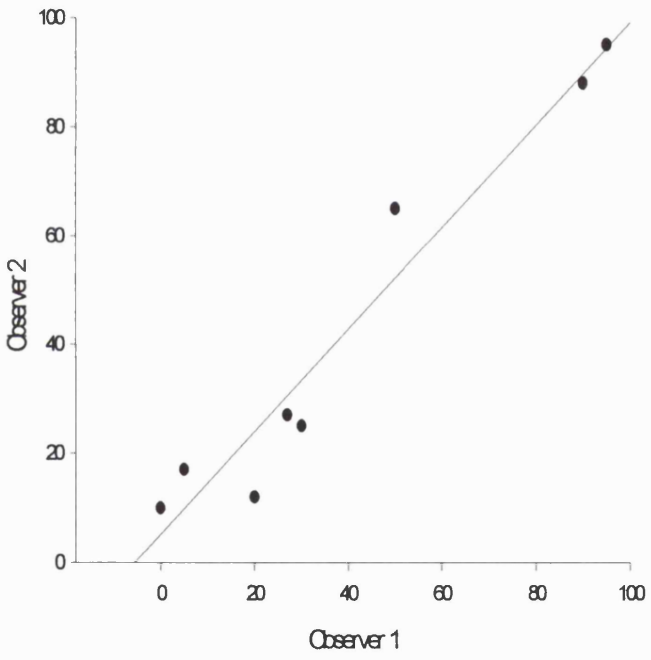


Figure 3.4 - Validation of data analysis
Two observers estimated separately the percentage of epithelium on eight slides using a grid overlay technique. A regression analysis was performed on the results which gave an R value of 0.97, demonstrating a good correlation.

immature epithelium was also calculated using this technique. Immature epithelium consisted of keratinocytes which had not yet reached the wound surface but were still migrating through the Integra®.

3.4.2. Qualitative analysis

Fibrin glue was well absorbed into the Integra® matrix. Keratinocytes can be seen throughout the Integra® on histological section of a specimen prior to implantation **(Figure 3.5)**.

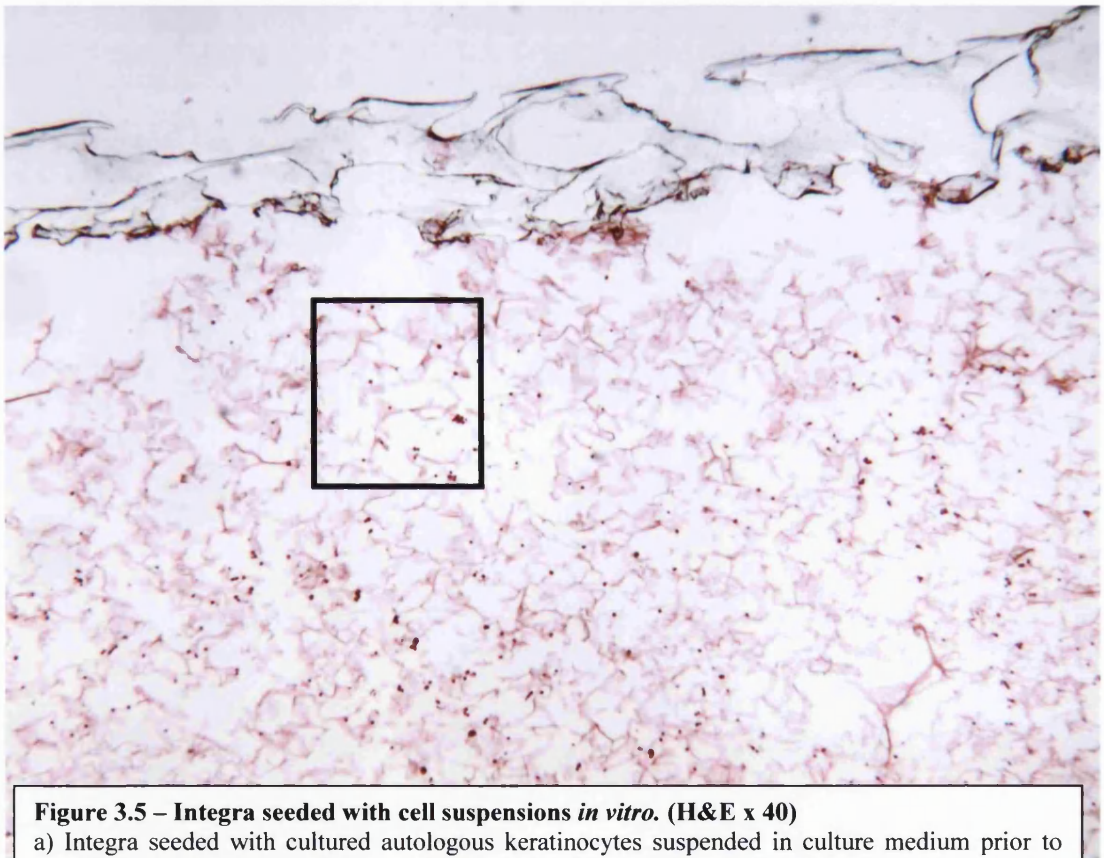


Figure 3.5 – Integra seeded with cell suspensions *in vitro.* (H&E x 40)
a) Integra seeded with cultured autologous keratinocytes suspended in culture medium prior to grafting. The silicone can be seen at the top of the photograph and the collagen matrix is visible as pink interlocking strands. The cells are just visible at this magnification. The square area is seen at high power overleaf in figure 3.6b.

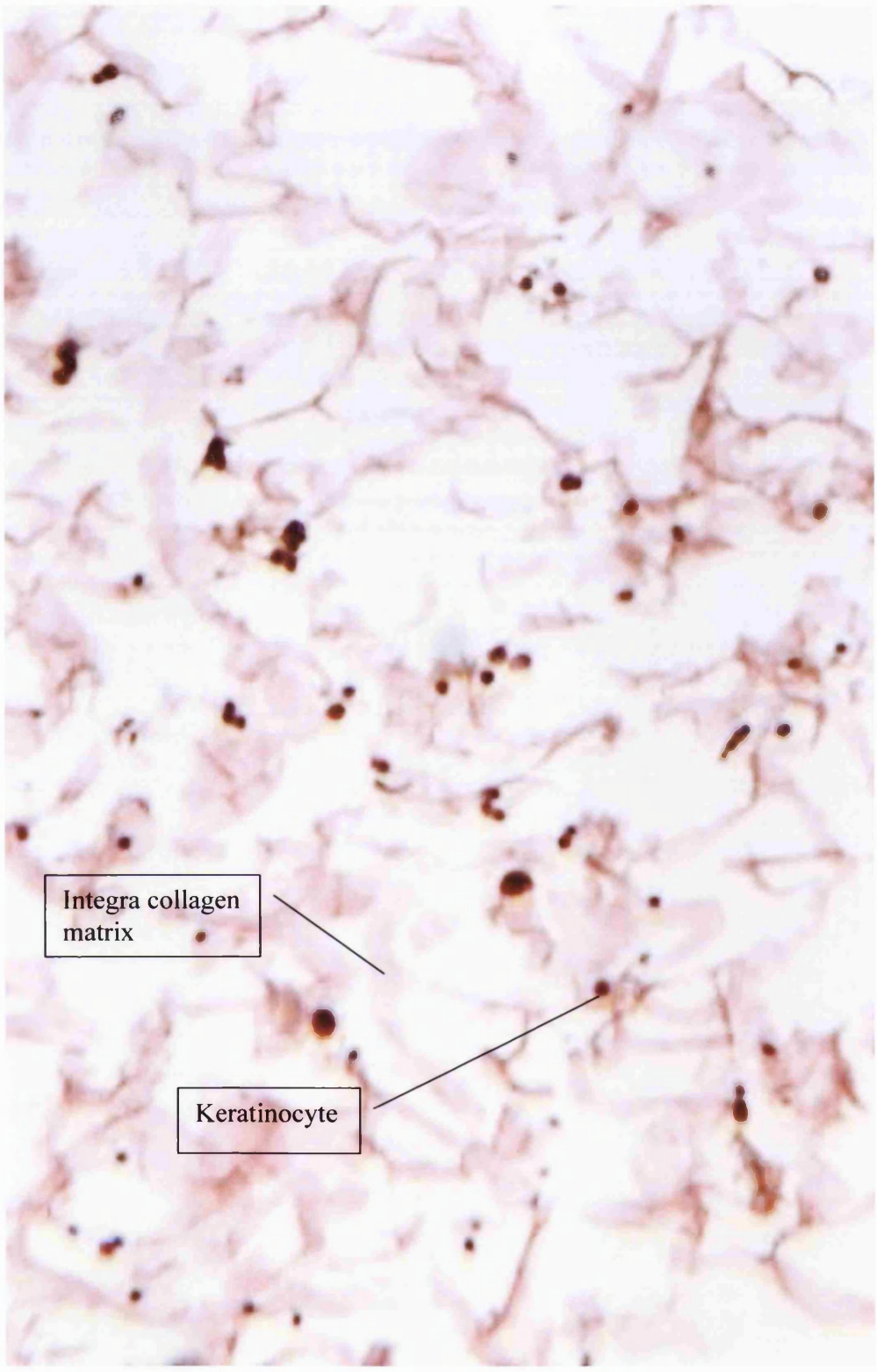


Figure 3.5 – Integra seeded with cell suspensions *in vitro*. (H&E x 100)
b) Integra seeded with cultured autologous keratinocytes suspended in culture medium prior to grafting.

The animals tolerated the procedure well with no loss of wound numbers from the study. Most grafts adhered well to the wounds with no significant loss of Integra® **(Figure 3.6)**. Any silicone remaining on the wound was removed from

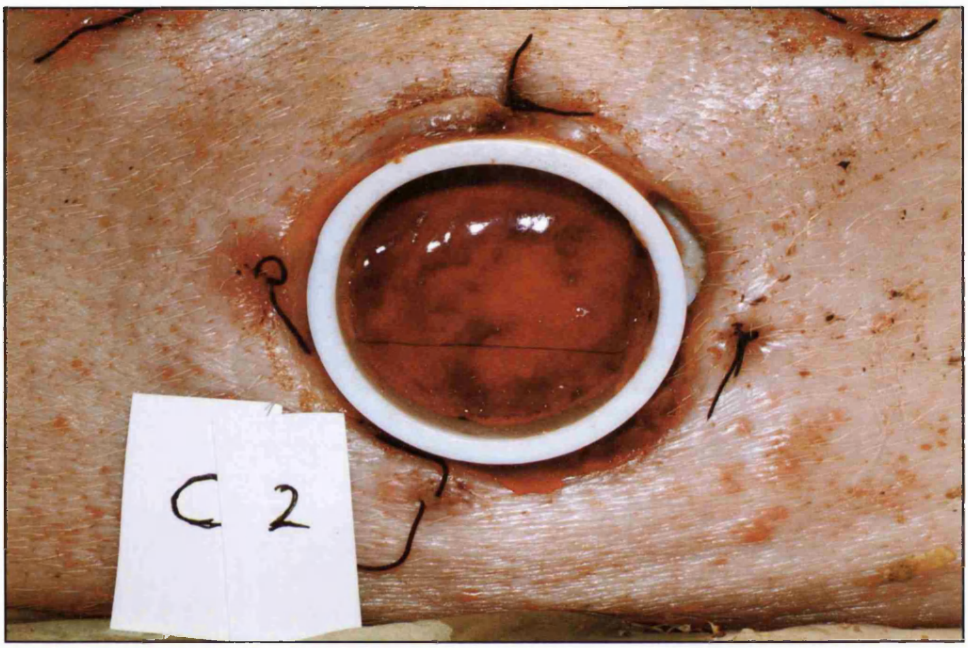


Figure 3.6 - Integra® in wound at 1 week
The clinical take rate of the Integra was good, with complete take in most of the wounds.

the Integra® at day 14 at which time no epithelium could be seen in the control wounds **(Figure 3.7a).** In the wounds seeded with keratinocytes a variable percentage of epidermal cover was observed **(Figure 3.7b)**, though no attempt was made to estimate this macroscopically.

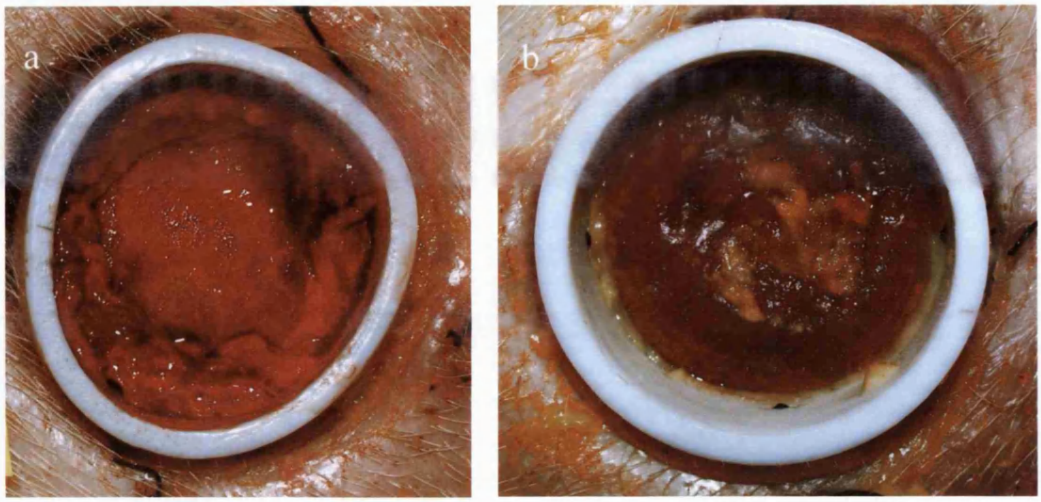


Figure 3.7 – Wounds at three weeks

a) Control wound grafted with Integra® only at three weeks. No epithelium has formed on the Integra®, and granulation tissue is beginning to form around the periphery of the wound at the edges of the Integra®.

b) Wound grafted with Integra seeded with cultured autologous keratinocytes at three weeks. Epithelium has formed over most of the wound surface.

In the 15 wounds grafted with fibrin glue, two intra-operative observations were made. Firstly, the application of the Integra® impregnated with fibrin glue was technically much easier. This was due to the instant adhesion of the Integra® to the wound bed. This prevented any movement during suture of the graft. Secondly, the colour of the Integra® in the grafts secured with fibrin glue remained pale compared to the non-fibrin group **(Figure 3.8)** which tended to become red soon after application to the wound. This was observed for over an hour after surgery, after which the wounds had to be dressed. This was thought to be due to the haemostatic effect of the fibrin glue.

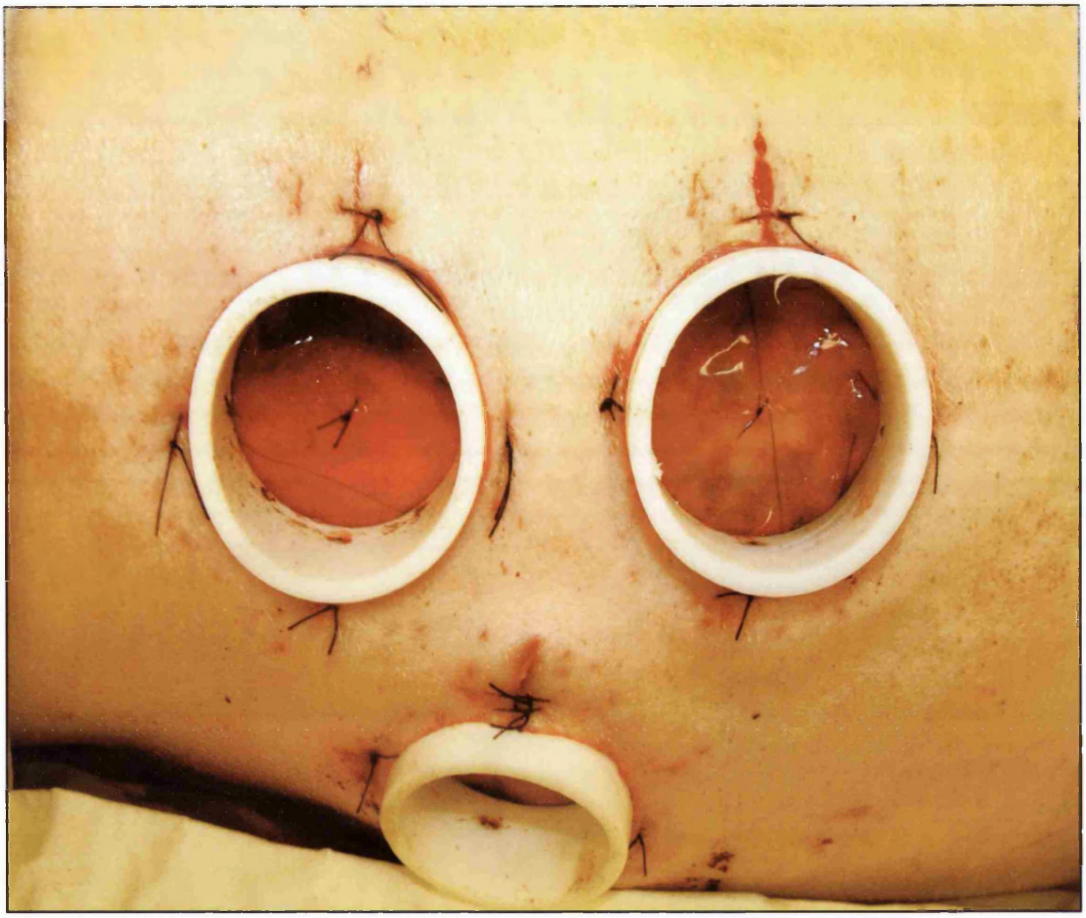


Figure 3.8 – Wounds immediately after grafting.
The wound on the left was grafted with Integra seeded with a suspension of cells in culture medium. The wound on the right was seeded with a suspension of cells in fibrin glue. This photograph was taken 30 minutes after graft application. The wound treated with fibrin glue has remained pale compared to the wound treated with culture medium, which displays some serosanguinous oozing into the Integra. This was a consistent finding throughout the study.

3.4.3. Histological findings

3.4.3.1. Integra® only

At one week the Integra® was sparsely populated with fibroblasts and inflammatory cells **(Figure 3.9)**. By week 2 **(Figure 3.10)** significant cellular infiltration had occurred with significant collagen deposition around the artificial dermal network. At 3 weeks there was evidence of extensive orderly collagen deposition with some resorption of the bovine collagen matrix **(Figure 3.11)**. Immunohistology to detect K14 shows no evidence of keratinocytes in any of the control wounds at any time point.

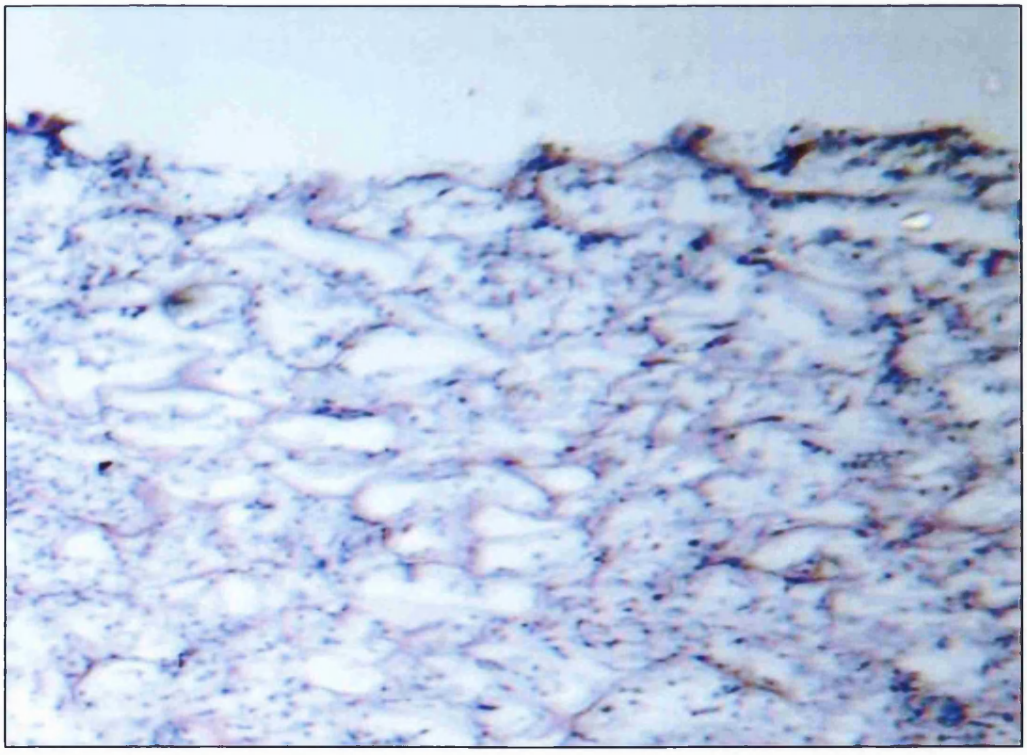


Figure 3.9 – Integra control wound at weeks 1 (H&E x 10)

At week 1 the Integra® is diffusely infiltrated with fibroblasts. There is very little collagen deposition.

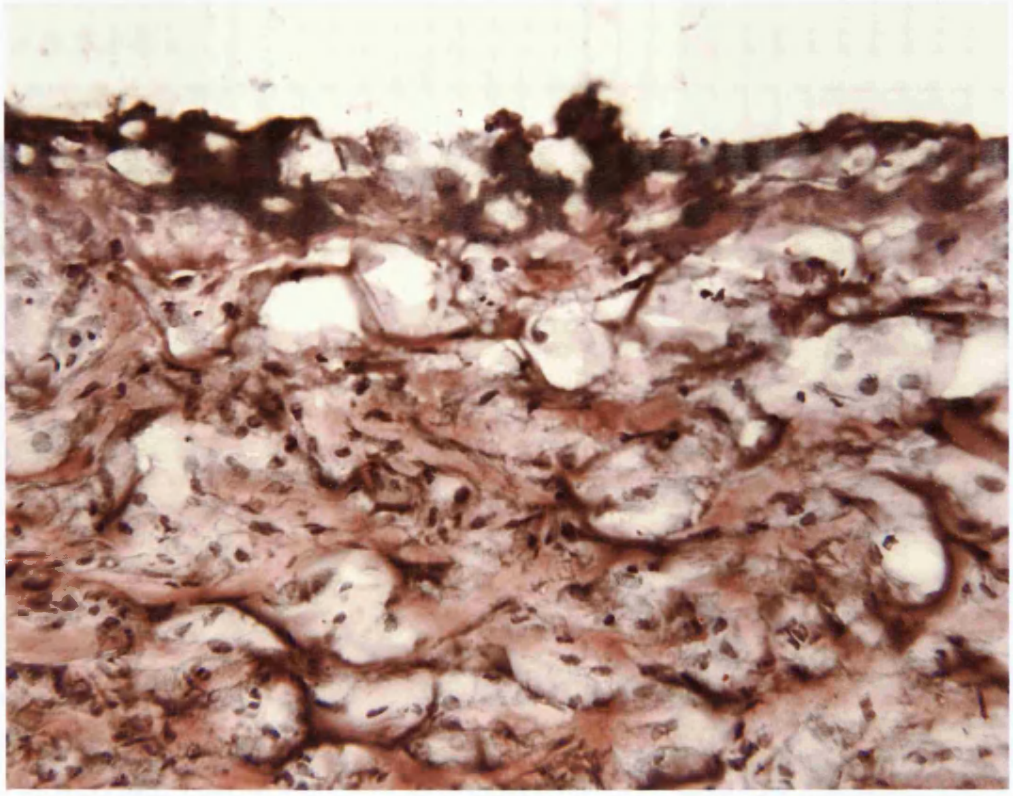


Figure 3.10 – Integra control wound at week 2 (H&E x40)
By the second week there are similar numbers of fibroblasts, but there is an orderly deposition of collagen around the matrix framework.

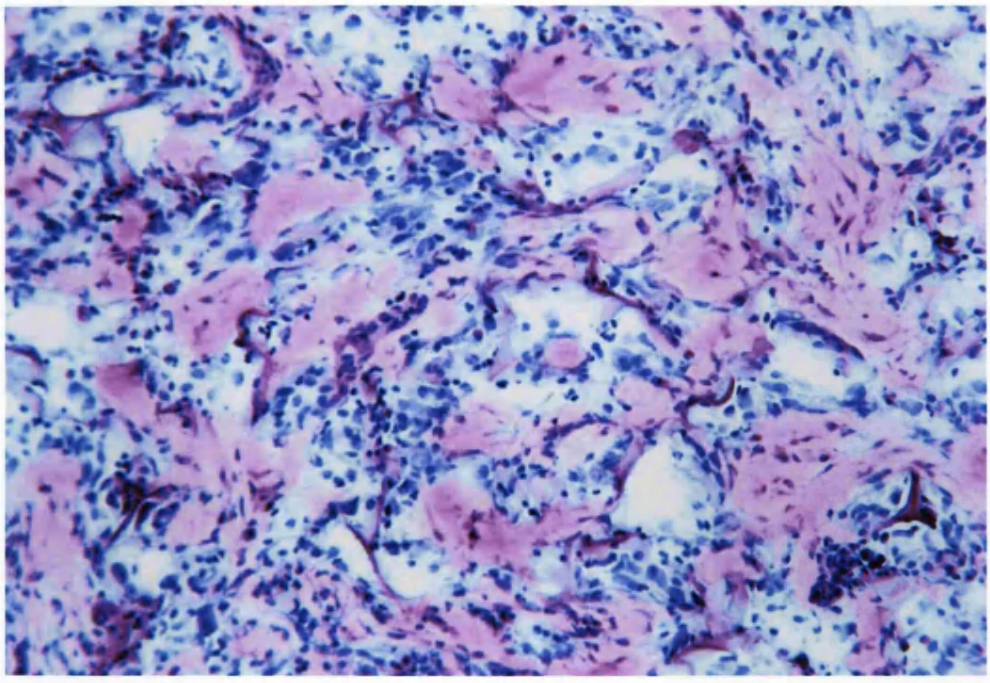


Figure 3.11 – Integra control wound at week 3 (H&E x40)
By the third week there is further orderly deposition of collagen around the matrix framework, with some resorption of the bovine matrix.

3.4.3.2. Integra® and fibrin glue

The sequence of wound colonisation was very similar to that for Integra® alone. The main difference was a slight increase in the rate of cellular infiltration of the Integra® the equivalent collagen production seems to occur slightly earlier, as seen at week 3 **(Figure 3.12** compared to **Figure 3.11)**. It is possible that the increased cellularity was due to infection of the Integra®. However, there was no clinical evidence of infection in any of the fibrin containing wounds biopsied at week 1 or 3, and infection usually results in loss of Integra® which has clearly not occurred here.

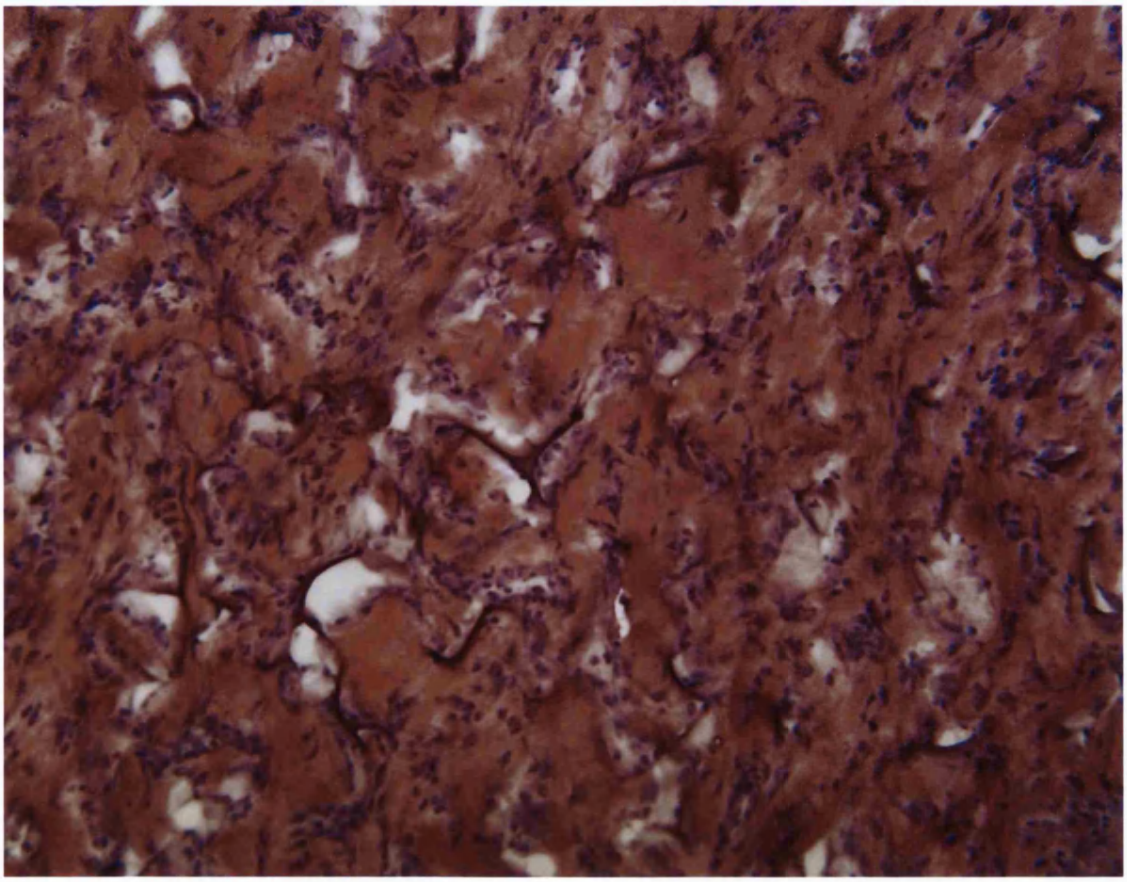


Figure 3.12 – Integra and fibrin glue control wound at weeks 3 (H&E x40)
By the third week there was slightly more deposition of collagen around the matrix framework when fibrin glue was used to secure the Integra.

3.4.3.3. Integra® and suspension of keratinocytes in culture medium

At one week the Integra® was uniformly infiltrated by proliferating keratinocytes **(Figure 3.13).** These were evenly distributed throughout the matrix in spherical accumulations consisting of 50-100 cells. These have previously been referred to as "keratomorula" (Kremer et al., 2000). Many of these keratinocyte accumulations had reached the surface of the Integra® by the second week, where they appeared to open out and form an epidermis. At week 3 the wounds had mature epidermis covering a significant percentage of the wound **(Figure 3.14)**. Immunohistology with antibodies to K14 and collagen VII showed a differentiated epidermis with collagen VII at the dermo-epidermal junction in a similar distribution to normal pig epidermis **(Figure 3.15)**. Some areas still contain immature epidermis, which has yet to reach the surface of the Integra®. These mature "keratomorula" were surrounded by a basement membrane as they migrate through the Integra® and often contained keratinised debris in their centre. (FIGURE 3.16) Interestingly, wherever mature epidermis had formed, "keratomorula" were no longer present in the dermis.

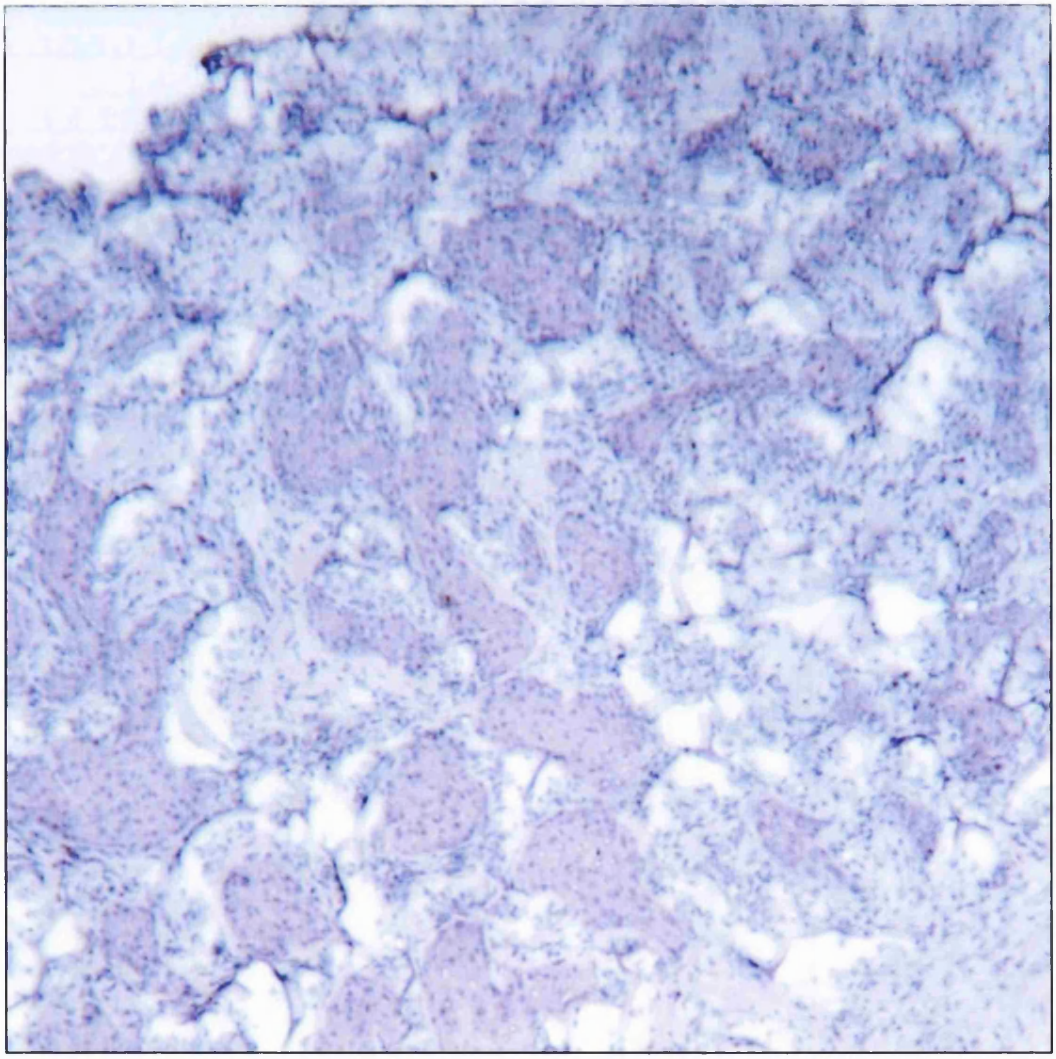


Figure 3.13 – H&E stain of a wound biopsy of Integra seeded with cultured autologous keratinocytes at 1 week (x10).
The Integra® is uniformly infiltrated by proliferating keratinocytes. These are evenly distributed throughout the matrix in spherical accumulations consisting of 50-100 cells. These have previously been referred to as "keratomorula".

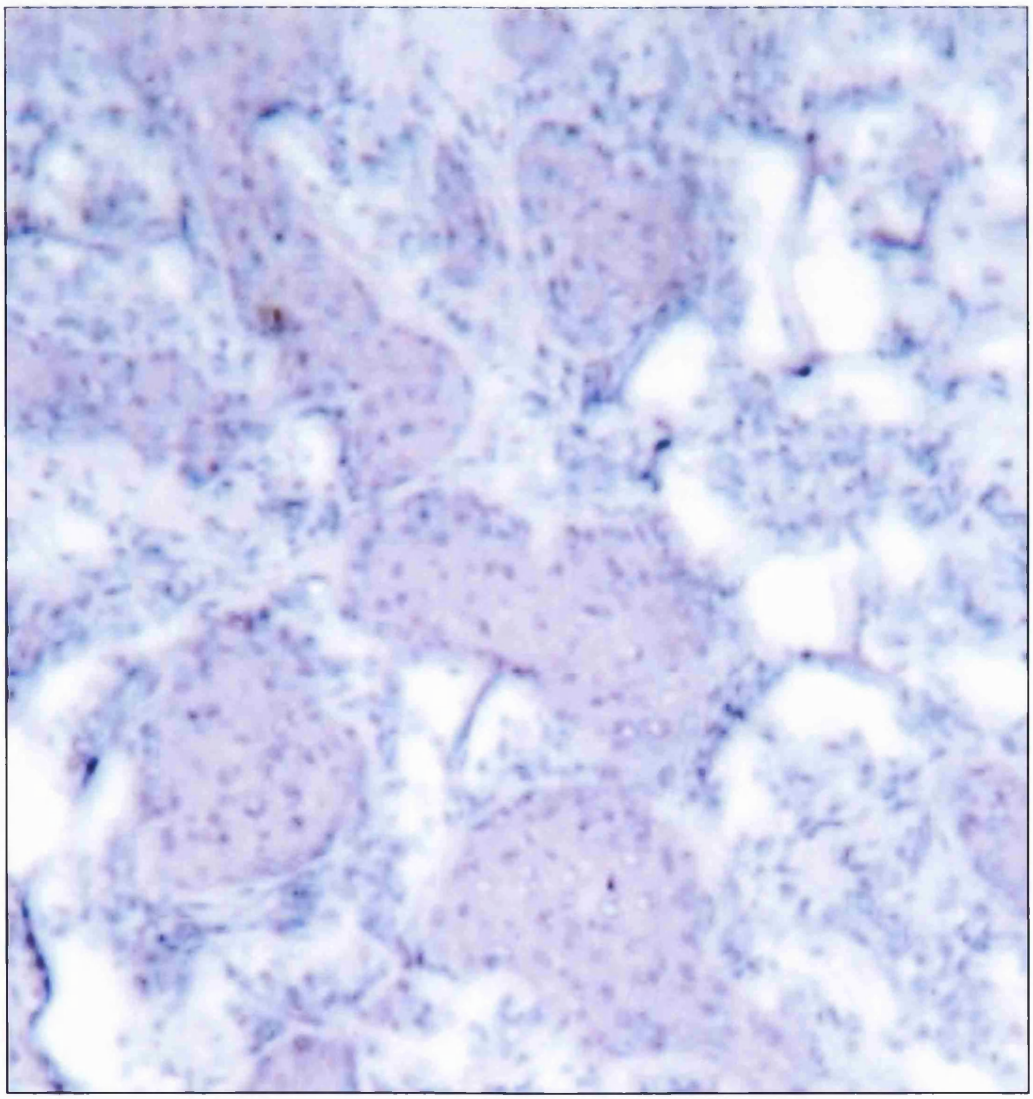


Figure 3.13 - H&E staining of wound biopsy with Integra® seeded with autologous keratinocytes at week 1 (x20).
Keratinocytes can be seen in a spherical accumulation consisting of 50-100 cells. This has previously been referred to as "keratomorula".

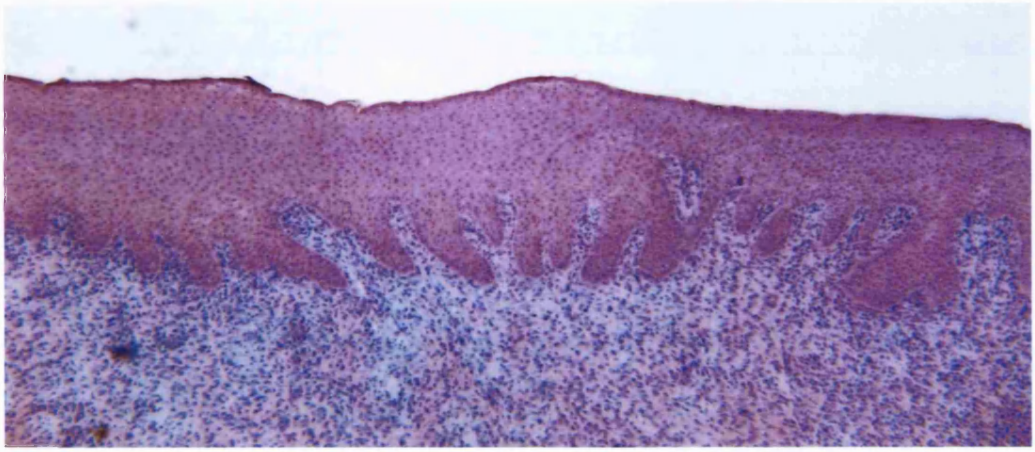


Figure 3.14 – Biopsy of Wound grafted with Integra® seeded with cultured autologous keratinocytes at 3 weeks.
H&E staining shows good quality epithelium on the wound surface.

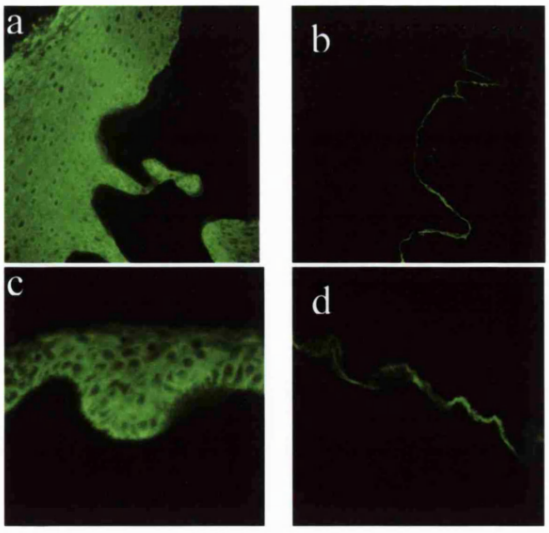


Figure 3.15 – Biopsy of Wound grafted with Integra® seeded with cultured autologous keratinocytes at 3 weeks.
a) Section stained with monoclonal antibodies to K14 (x100).
b) Section stained with monoclonal antibodies to collagen VII (x100).
The epithelium expressed keratin 14 while collagen VII was present at the dermo-epidermal junction.
c) and d) are the equivalent stains in normal pig skin.

3.4.3.4. Integra® and keratinocytes suspended in fibrin glue

The sequence of keratomorula formation and epidermal development when the cells were applied in fibrin was very similar to that seen for Integra® and keratinocytes alone. The main difference is that the keratinocyte accumulations have a more flattened morphology, as is best seen on K14 staining **(Figure 3.16)**.

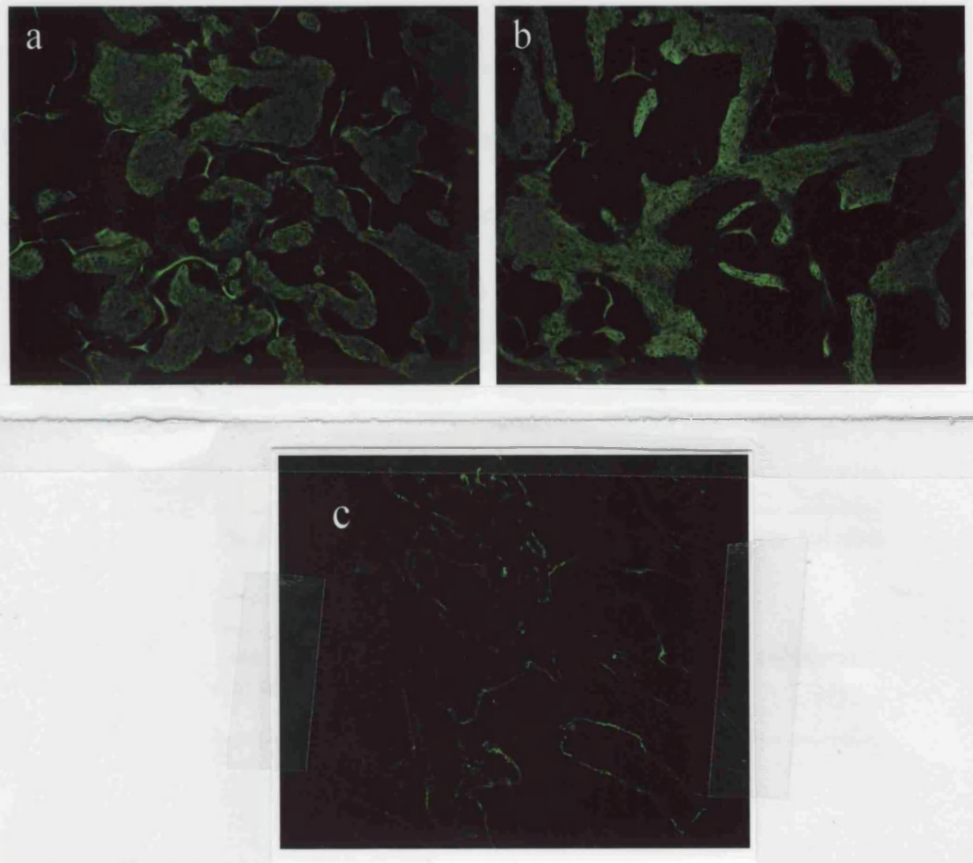


Figure 3.16 – Keratin 14 stain of Integra® seeded with cultured autologous keratinocytes at 1 week (x40).
a) Keratinocytes were seeded in a suspension of culture medium
b) Keratinocytes were seeded in a suspension of fibrin glue
The Integra® matrix can be seen amongst the keratinocyte collections as it autofluoresces. The keratinocytes seeded in fibrin glue have a more linear morphology.
c) Collagen VII stain of Integra® seeded with cultured autologous keratinocytes at 1 week

3.4.4 Quantitative analysis

3.4.4.1. Percentage of epithelial cover

There was no epithelium found in any of the 10 control wounds.

The percentage of keratinocytes within the assimilated matrix at consecutive weekly intervals is demonstrated in **Figure 3.20**. Initially the matrix contains a large number of keratinocytes, but by the fourth week the matrix contains very few. There was no statistical difference in the percentage of matrix containing keratinocytes in the group treated with or without fibrin glue (P=0.319).

Conversely, **Figure 3.21** demonstrates the percentage of epithelium covering the wounds at consecutive weekly intervals. The percentage of surface epithelium in the wounds grafted with Integra® seeded with cultured autologous keratinocytes increased with time in both groups. There was 42.1% epithelial cover in the fibrin group by three weeks, compared to 20.4% in the non-fibrin group. However, there was no statistical difference in the percentage of surface epithelium present at week 3 in the fibrin versus the non-fibrin group (P=0.097), because of large experimental variations.

Therefore, in both the fibrin and non-fibrin groups, there is a general trend consisting of a gradual reduction in the percentage of keratinocytes within the assimilated matrix, and an increase in the percentage of mature epithelium at consecutive weekly intervals. This confirms that the keratinocytes migrate

through the Integra® to the surface of the matrix. The macroscopic appearance of the epithelium produced on the wound surface is shown in **Figure 3.22**.

3.4.4.2. Percentage of Integra® "take"

The overall "take" of the Integra® was 86% (average of all four time points). The rate for Integra® alone was 90%, and that for Integra® applied with fibrin glue was 80%. The "take" rate for Integra® and a cell suspension of keratinocytes was 83%, whilst the "take" rate for Integra® and keratinocytes suspended in fibrin glue was 92%.

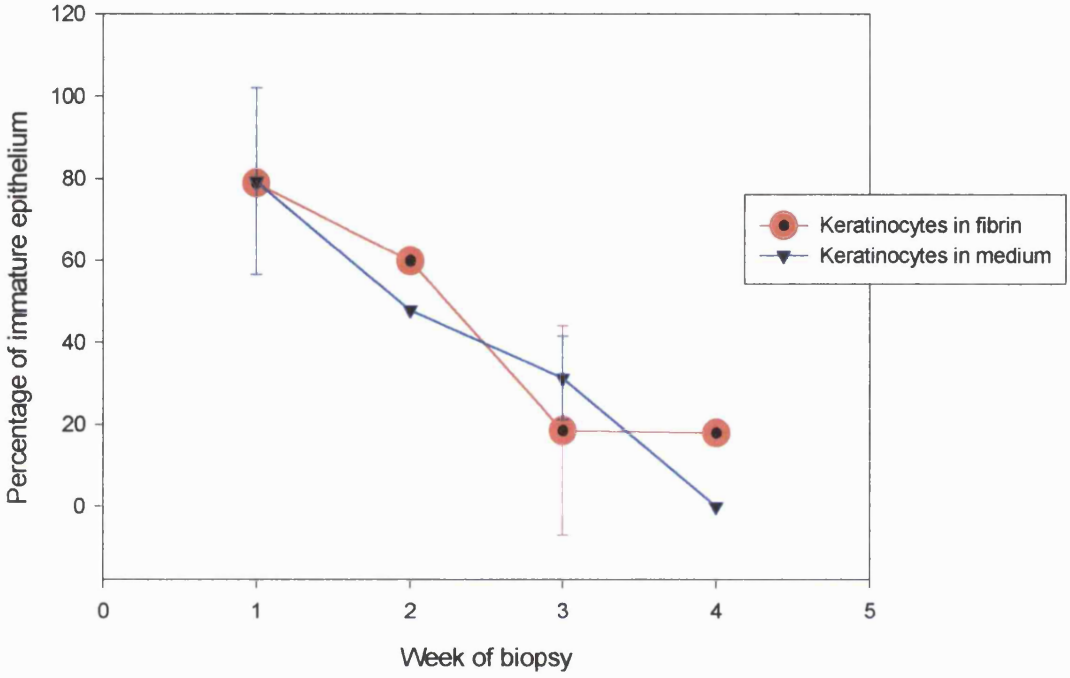


Figure 3.20 – Quantification of immature epithelium.
Graph of percentage of immature epithelium against time. Immature epithelium consisted of keratinocytes, which had not yet reached the wound surface but were still migrating through the Integra®. The difference between the percentage of immature epithelium in the wounds seeded with cells either with or without fibrin glue was tested at 3 weeks and was not significant (P=0.319). (N=1,1,7,1 for fibrin group and N= 3,1,5,1 for non fibrin group)

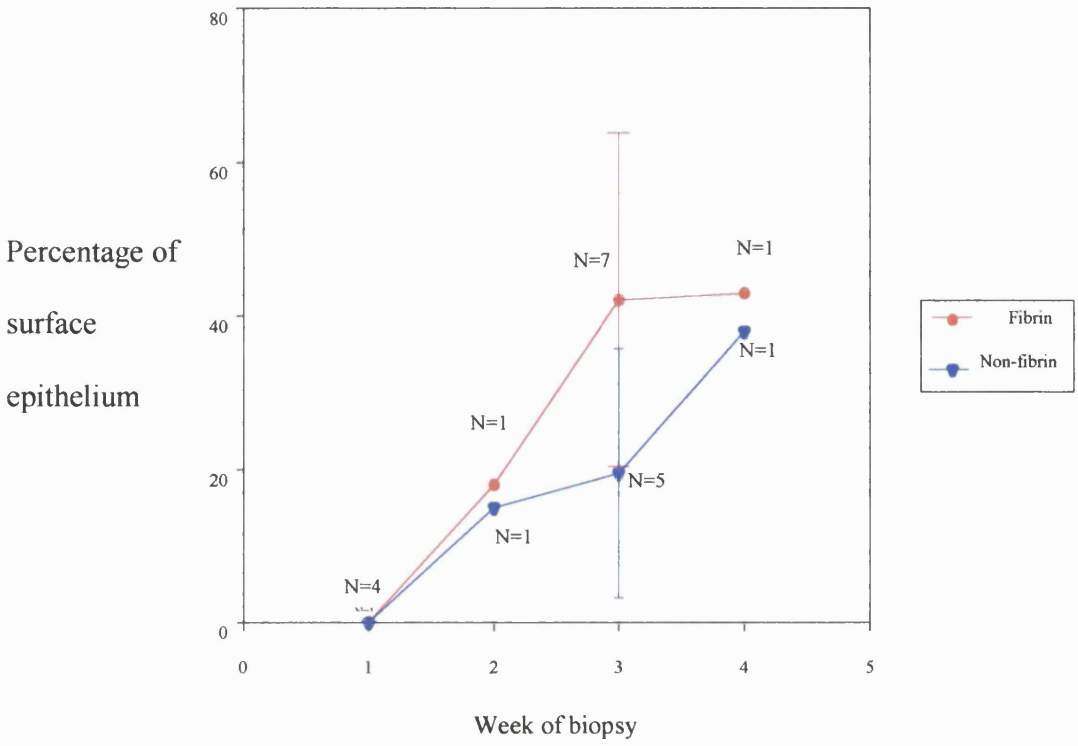


Figure 3.21 – Quantification of mature epithelium.
Graph of percentage of surface epithelium against time. The percentage of surface epithelium in the wounds grafted with Integra® seeded with cultured autologous keratinocytes increased with time in both groups. There was a mean of 42.1% epithelial cover in the fibrin group by three weeks, compared to a mean of 20.4% in the non-fibrin group. However, this difference between the percentage of mature epithelium in the wounds seeded with cells either with or without fibrin glue was tested and was not significant (P=0.097), because of large experimental variations.

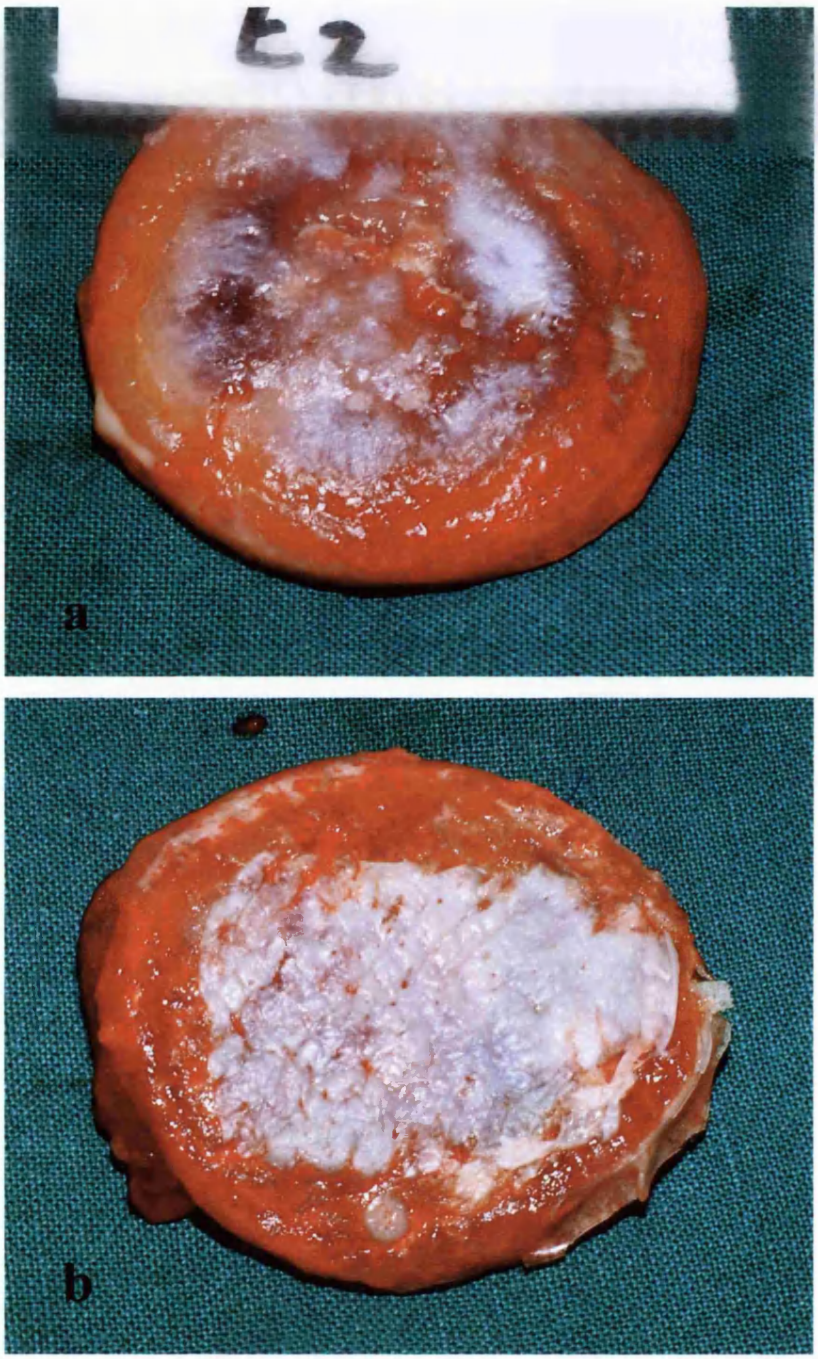


Figure 3.22 – Macroscopic appearance of wound biopsies
a) Total wound biopsy of pig wound at three weeks, which had been grafted with Integra seeded with cultured autologous keratinocytes. A good quality epithelium has been produced over the wound surface
b) Total wound biopsy of pig wound at three weeks, which had been grafted with a split skin graft. This biopsy was from another experiment and is included here for comparison. The split gskin graft was from within a wound chamber in identical conditions to the specimen in a).

3.5 Discussion

Epithelium was not found in any of the ten control wounds, which were covered with Integra® only. As PTFE chambers had prevented any epithelial migration into the control wounds, any surface epithelium found in the other wounds can be assumed to have arisen from the cultured autologous keratinocytes seeded into the Integra®.

Cultured autologous keratinocytes seeded into Integra® produced a mean of 20.4% surface epithelium by three weeks. The same number of cultured autologous keratinocytes suspended in fibrin glue and seeded into Integra® produced a mean of 42.1% surface epithelium at the same time point. Wound keratinocytes express functionally active integrin receptors for fibronectin and can pave the wound surface with a provisional matrix and express cell surface receptors that facilitate their migration across this matrix (Clark, 1990). Many fibrin glues contain fibronectin, therefore one could expect fibrin glue to improve the rate of keratinocyte transit through Integra®. Although this trend towards an increase in the percentage of surface epithelium in the fibrin group was not statistically significant, these results have demonstrated that fibrin glue can be used as a delivery system for seeding Integra® with cultured autologous keratinocytes. The fibrin glue made suture of the Integra® technically simpler, and had a subjective haemostatic effect, as observed by the reduction of sero-sanguinous exudate in the Integra® (Figure 3.8).

This technique also has the theoretical advantage of improved Integra® take rates and a reduction in intra-operative blood loss which has been seen with the use of fibrin glue in conventional split skin grafts (Currie et al., 2001). In our study we attempted to measure the "take rate" of the Integra® histologically, but found no significant difference between the fibrin and non-fibrin groups. In this pig model a take rate of 95% has been demonstrated by the use of meticulous surgical technique, antibacterial agents and pressure dressings (Grant, 2001). The addition of fibrin glue is unlikely to have a significant enhancement with such high take rates already. However, in burns patients, the take rate of Integra® can be lower due to bacterial colonisation and poor wound healing. In this scenario any improvement in the take rate of Integra® associated with the use of fibrin glue could be significant. The application of Integra® to sites where it is difficult to immobilise patient movements, could significantly benefit from fibrin glue mediated adhesion. This is currently being examined in a non-chambered pig wound model in our unit.

Several authors have expressed concern over the formation of keratinocyte cysts within the Integra® matrix. However, these experiments demonstrate a linear nature in the relationship between the percentage of immature epithelium within the matrix and the time of biopsy. This suggests that the keratinocytes continue to migrate through the matrix and only differentiate when they reach the surface. However, this may be a drawback with the use of this technique, and long-term studies to assess the quality of the epithelium and the neodermis produced using this technique are needed.

There are some disadvantages with the use of fibrin glue. Despite donor selection, viral inactivation and product screening, commercial fibrin glue still has a theoretical risk of viral transmission. However, these theoretical risks would be equilibrated in burn patients who may receive numerous blood transfusions, and any reduction in operative bleeding will reduce the transfusion requirements. Any bovine components likewise carry the risk of prion transmission and anaphylaxis. Autologous fibrin glue avoids the risk of infection, but few centres are equipped for its manufacture. In the future recombinant fibrinogen and other components may provide the solution.

This study has shown that fibrin glue does not have a detrimental effect on Integra® "take" rate, and we have shown that fibrinogen solution can be used to seed the Integra® matrix with cultured autologous cells. Clinical studies to evaluate the effect of fibrin glue on the "take" of seeded and unseeded Integra® in burn patients are now warranted. In the future a one step technique to provide a dermis and an epidermis may be possible using these techniques.

CHAPTER 4

A COMPARISON OF INTEGRA® SEEDED WITH CULTURED AND NON-CULTURED KERATINOCYTES: THE SELECTIVE ADVANTAGE OF KERATINOCYTE CULTURE

4.1 Introduction

Many groups are working on the development of alternative systems for delivery of cultured autologous keratinocytes in the hope that this may improve the take and the quality of the resulting epidermis. A common approach is to use a dermal analogue in combination with cultured autologous keratinocytes. The dermal analogues most commonly used include cadaver de-epithelialised dermis (Ben-Bassat et al., 1992;Ojeh et al., 2001) and collagen glycosaminoglycan matrices with (Coulomb et al., 1998;Boyce et al., 1995) or without(Kremer et al., 2000) autologous fibroblasts.

When cultured autologous keratinocytes are used to seed Integra® or similar collagen glycosaminoglycan matrices, the aim is to provide a dermal layer on which an epidermis will form *in vivo* without the need for autologous split skin grafts. Three basic strategies have been tried. Firstly, covering the Integra® neo-dermis with sheets of cultured autologous keratinocytes after it has matured *in vivo* (Hafemann et al., 1999;Pandya et al., 1998). Secondly, culturing collagen matrices with autologous keratinocytes such that an epidermal layer is formed *in vitro* (Boyce, 1998;Boyce et al., 1999) and is subsequently grafted to mature bio-integrated Integra® *in vivo*; or finally, as Integra® matrices seeded with cultured autologous keratinocytes (as demonstrated in Chapter 3) which form an epidermis *in vivo* simultaneously with the formation of the neo-dermis (Butler et al., 1998;Compton et al., 1998).

Culturing cells to a non-confluent state *in vitro*, and then delivering them to the wound centrifuged into an Integra®-like collagen glycosaminoglycan matrix has been demonstrated to form a mature epidermis over the dermal regeneration template (Butler et al., 1998;Compton et al., 1998;Kremer et al., 2000). This may confer several advantages over other methods. It provides a one step procedure to deliver both a dermal and epidermal layer to the wounds. In patients with major burns and limited autologous split skin available for grafting, sufficient cells could be cultured in a few weeks to allow a one step wound closure. A temporary wound closure would be needed during the intervening culture time. However, a better solution may be to seed the matrix on the day of burn wound excision with a suspension of **non-cultured** autologous keratinocytes which have been isolated from split skin grafts taken from non-burned areas. This would avoid the need for a temporary wound cover. If this technique produced a good quality cover it would allow a simple, one-step procedure which does not require complex culture techniques or expensive and inconvenient centrifugation.

Non-cultured autologous keratinocytes have been shown to behave in a similar manner to cultured autologous keratinocytes when centrifuged into a porous collagen-glycosaminoglycan (C-GAG) matrix (Butler et al., 1999). However, the non-cultured cells produced only half as much epithelium when compared with the cultured cells at the same time point.

4.2 Study objectives

The aim of this study was to investigate the effect of seeding **non-cultured** autologous keratinocytes into the commercially available dermal regeneration template Integra®. The quality of the epidermis and dermis produced was assessed.

4.3 Experimental design

4.3.1. Animals

Twenty-two full thickness pig wounds were created in 4 animals. Six wounds were made on the flanks of each animal, down to skeletal muscle. The PTFE chambers were inserted as (cf. 2.2.15). This prevented re-epithelialisation from the wound margins and prevented the wounds from contracting. Thirteen wounds were grafted with Integra® seeded with **non-cultured** autologous keratinocytes. One wound from this group was removed at week one, and one at week two, for histological analysis. Five wounds were grafted with Integra® seeded with **cultured** autologous keratinocytes (positive control) and four wounds were grafted with Integra® alone (negative control). The remaining wounds were all harvested at three weeks.

4.3.2. Keratinocyte isolation and culture

Pig keratinocytes were isolated from split skin grafts according to the protocol already described (cf. 2.2.5). The autologous keratinocytes were cultured

through four passages over a three week period, and then harvested from culture whilst still in a pre-confluent state (cf. 2.2.6). The autologous keratinocytes were counted with a haemocytometer, then centrifuged and re-suspended in keratinocyte growth medium in a concentration of 6.25 x 10^6 keratinocytes per ml for seeding into the Integra®.

On the day before the wound chamber insertion a second split skin graft was harvested from the flank of each pig in a position caudal to the site of wound chamber insertion. Keratinocytes were isolated from the biopsy (cf. 2.2.5), except these keratinocytes were not plated onto collagen coated flasks for culture with 3T3 feeder cells. Instead, the keratinocytes were stored in non-coated flasks as a suspension in keratinocyte growth medium. This prevented them from attaching and dividing, but maintained cell viability (measured by tryp an blue uptake). The following day this suspension of non-cultured autologous keratinocytes was centrifuged and re-suspended in keratinocyte growth medium in a concentration of 6.25 x 10^6 keratinocytes per ml for seeding into the Integra®.

4.3.3 Preparation of grafts

The grafts were prepared as already described (cf. 2.2.16). The cultured and non-cultured keratinocytes were both stored in keratinocyte culture medium prior to their delivery into the Integra®. Keratinocytes were delivered into Integra® by dripping a suspension of cells onto the non-siliconised surface. The average seeding density was $5x10^5$ keratinocytes per cm^2. Cell viability at this

time was 62% and 65% in the cultured and non-cultured groups respectively, as assessed by a tryp an blue exclusion test.

4.3.4 Analysis of results

A single excision biopsy was harvested from the non-cultured keratinocyte group at week one, and again at week two. The remaining wounds were all harvested at three weeks. The dissection was performed to include the most superficial part of the underlying muscle. The chamber was removed and the residual wound was dressed with saline soaked gauze. The excision biopsy was then cut into quadrants **(cf. Figure 3.3)** and the four indicated faces were prepared for histology and immunohistochemistry (cf. 2.2.18-21).

4.3.5 Estimation of percentage of epithelial wound cover

The percentage of epithelial cover was calculated by examination of histological sections. The percentage of mature epithelium was calculated by grid overlay on each slide (cf. 3.3.6) and the mean percentage of epithelial cover calculated for each wound. The percentage of immature epithelium was also calculated using this technique. Immature epithelium consisted of keratinocytes which had not yet reached the wound surface but were still present within the Integra®. Statistical analysis was performed using the program Sigma Stat version 2.0 (Jandel Scientific).

4.3.6 Immunohistochemical detection of cytokeratins and collagen

Mouse monoclonal antibodies were supplied by Professor Irene Leigh (The Royal London Hospital). The presence of keratinocytes was confirmed using mouse monoclonal antibody LL001 to keratin 14. This antibody was raised to human keratin antigens, but has been shown to cross react with porcine skin where it is present in all basal and suprabasal keratinocytes, along with its pair, keratin 5 (Bevan et al., 1997). LH7.2, an anti-human collagen VII mouse monoclonal antibody which cross reacts with porcine type VII collagen, was used to confirm the location of the basal epidermal dermal junction. Differentiation was identified in keratinocytes using mouse monoclonal antibody LHP2 an anti-human keratin 10 antibody which cross reacts with porcine keratin 10. The pair of keratins, keratin 1 and keratin 10, are produced in the superficial epidermis and are markers of differentiation. Keratin 1 and keratin 10 are not produced *in vitro* during cell culture. Frozen sections were fixed for 20 minutes in methanol and acetone (1:1), rinsed in phosphate buffered saline (PBS), and incubated with primary antibodies for one hour. They were then incubated with the secondary antibody (sheep anti-mouse fluorescein conjugated antibody F6257, Sigma) for 45 minutes. The nuclei were then stained with propidium iodide for 20 seconds.

4.4.1. Qualitative assessment of epithelialisation in wounds seeded with Integra® containing cultured and non-cultured autologous keratinocytes.

In the negative control wounds, where Integra® had been grafted in the absence of keratinocyte seeding (n=4), no epithelium was seen on multiple histological sections. In the wounds grafted with Integra® seeded with cultured autologous keratinocytes (positive controls) collections of keratinocytes could be seen in the Integra® matrix from week one, and by week three most of the keratinocytes had moved to the surface of the Integra®. **Figure 4.1b** shows a typical section of epithelial cover on a wound grafted with cultured keratinocyte seeded Integra® at three weeks.

At one week the Integra® seeded with **non-cultured** keratinocytes was also uniformly infiltrated by proliferating keratinocytes. These were evenly distributed throughout the matrix in spherical accumulations consisting of 50-100 cells. **Figure 4.1a** shows the even distribution of these non-cultured cells throughout the matrix. However, by the second week many of the non-cultured keratinocytes had started to differentiate and none of them had reached the surface of the Integra®. **Figure 4.1c** demonstrates the appearance of keratinocyte cysts throughout the matrix at this time period, and **Figure 4.1d** demonstrates an increase in size of the cysts by the third week, with extensive cell debris at their core. These cysts had not migrated any closer to the wound surface.

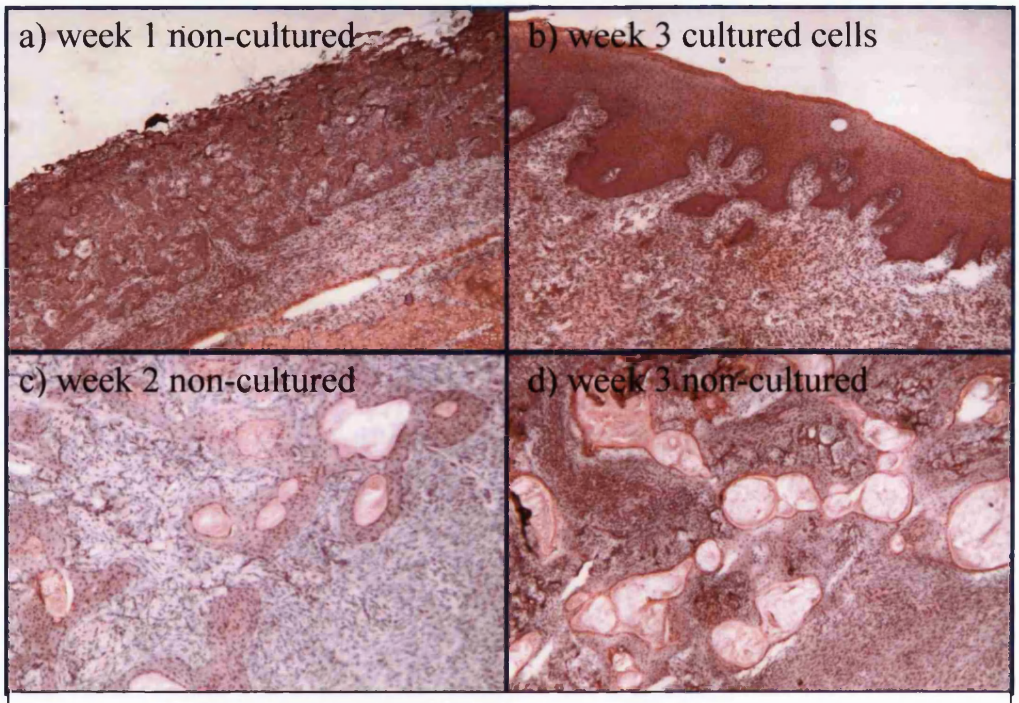


Figure 4.1 – H&E sections of wound biopsies

a) Non-cultured autologous keratinocytes seeded into Integra® at one week. The keratinocytes can be seen evenly distributed throughout the matrix (x 10).

b) Cultured autologous keratinocytes seeded into Integra® at three weeks. The cultured keratinocytes have migrated to the surface of the Integra® and produced a squamous epithelium (x 10).

c) At two weeks non-cultured autologous keratinocytes can still be seen in the Integra® matrix. They have formed small cysts with "keratin" deposited in the centre (x40).

d) At three weeks the cysts which were apparent at two weeks have enlarged and the non-cultured keratinocytes have failed to migrate through the Integra® matrix in a similar manner to the cultured keratinocytes (see b) (x 40).

4.4.2. Quantitative assessment of epithelialisation in wounds seeded with Integra® containing cultured and non-cultured autologous keratinocytes.

Five wounds were grafted with Integra® seeded with **cultured** autologous keratinocytes. In this group there was a mean epithelial cover of 24.6% at three weeks (n=5). Eleven wounds were grafted with Integra® seeded with **non-cultured** autologous keratinocytes and the mean epithelial cover was only 6.7% at three weeks (n=11). **Figure 4.2a** shows that there was a statistically significant difference in the area of epithelial wound cover between the cultured cell group and the non-cultured cell group (P=0.009).

The percentage of epithelium still within the Integra® matrix at three weeks in the control group of **cultured** cells was 13.2% (n=5). In the **non-cultured** cell group the percentage of epithelium still within the Integra® matrix at three weeks was 48.5%, mostly in the form of differentiated keratinocyte cysts (n=11). However, the difference in the mean values of the two groups was not great enough to reject the possibility that the difference is due to random sampling variability (P=0.097). This is shown graphically in **Figure 4.2b.**

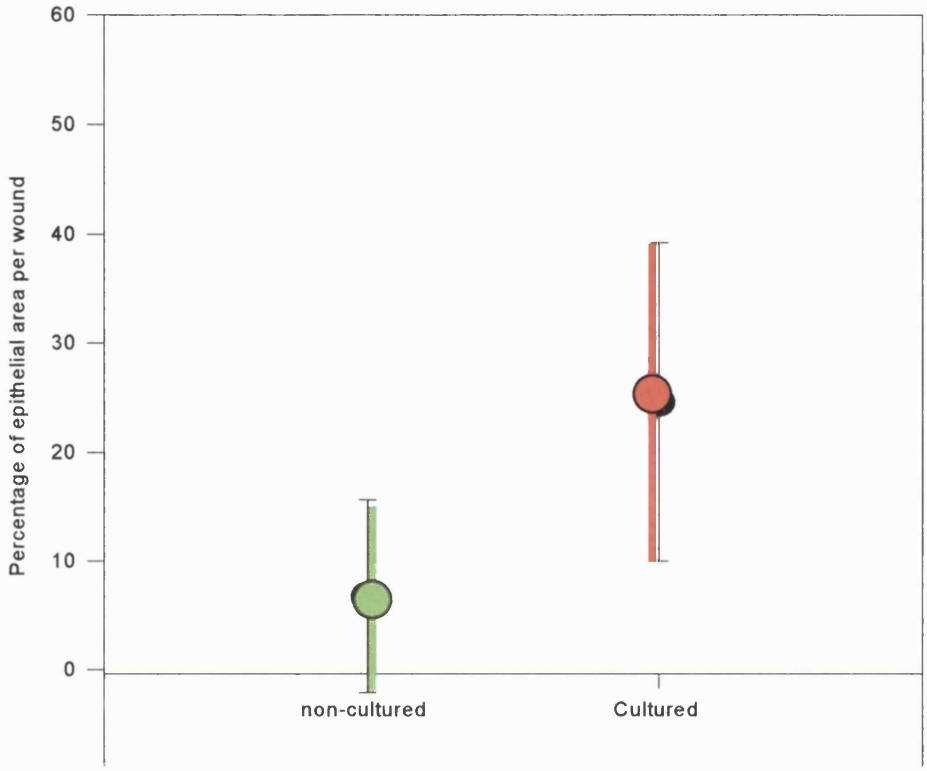


Figure 4.2a – Graph showing the percentage of epithelial area at three weeks in the cultured keratinocyte and non-cultured keratinocyte groups after seeding into Integra®.
In the cultured keratinocyte group there was a mean epithelial cover per wound of 24.6% (n=5). In the non-cultured group there was a mean of only 6.7% epithelial cover per wound (n=11). There was a statistically significant difference in the area of epithelial wound cover between the two groups (t-test P=0.009). One SD shown as bar.

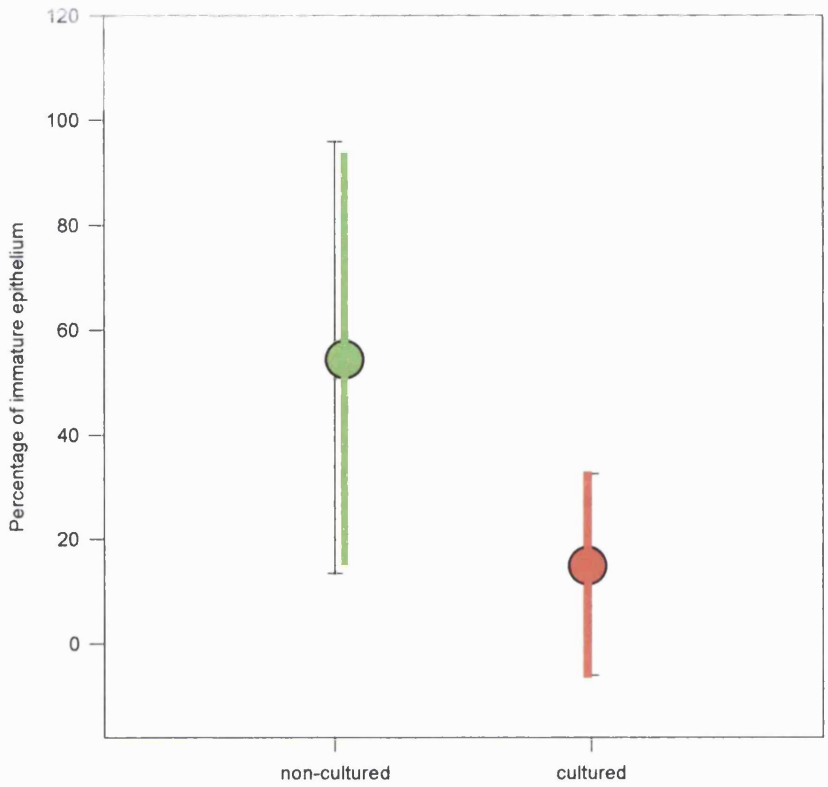


Figure 4.2b – Graph showing the percentage of keratinocytes within the Integra® matrix at three weeks in the cultured keratinocyte and non-cultured keratinocyte groups after seeding into Integra®.
The percentage of epithelial cells still within the Integra® matrix at three weeks in the control group of cultured cells was 13.2% (n=5). However in the non-cultured cell group the percentage of epithelial cells still within the Integra® matrix at three weeks was 48.5% (n=11). However, the difference in the mean values of the two groups was not great enough to reject the possibility that the difference is due to random sampling variability (t-test P=0.097). One SD shown as bar.

4.4.3. Immunohistological assessment of epithelialisation in wounds seeded with Integra® containing cultured and non-cultured autologous keratinocytes.

Figure 4.3 shows that the epithelium produced by the **cultured** autologous keratinocytes expressed keratin 14 and collagen VII in a similar distribution to normal pig skin. When **non-cultured** cells were seeded into the Integra® very little epithelium was produced, as described above. Instead, many keratinocyte

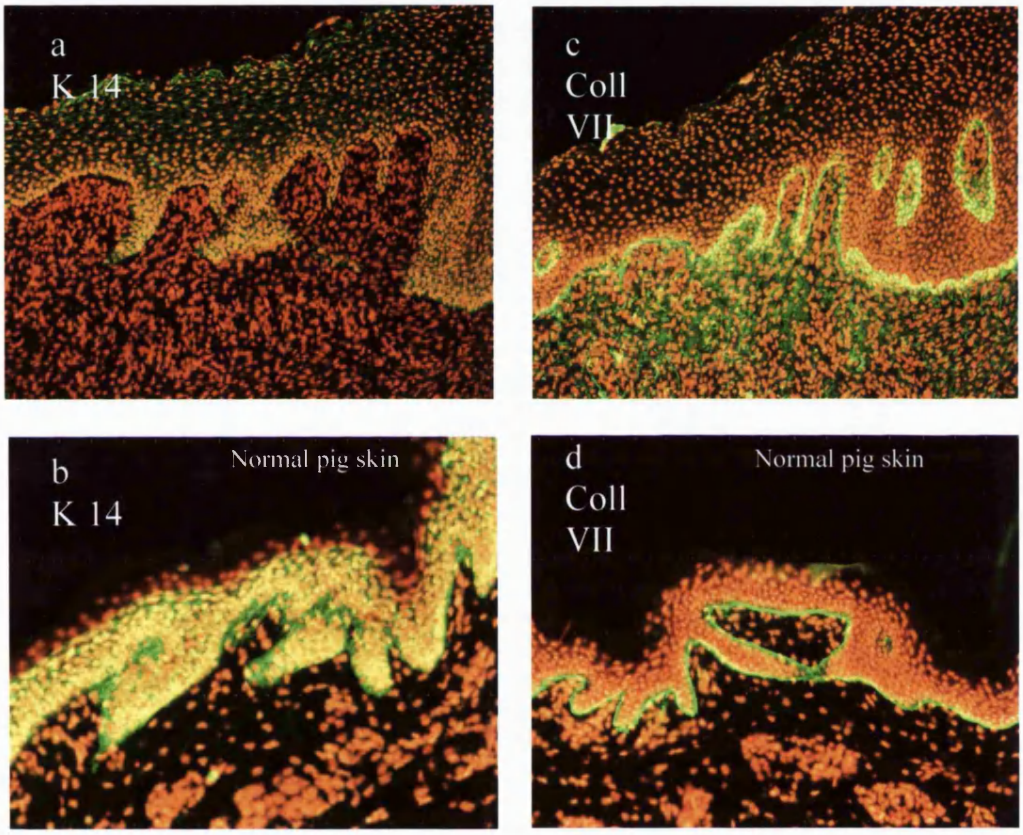


Figure 4.3 – Immunohistochemistry of epithelium produced by cultured autologous keratinocytes when seeded into Integra® artificial skin. (x40)
Histological sections a) and c) are from Integra® seeded with cultured autologous keratinocytes and transplanted into a pig wound, biopsied at three weeks. Histological sections b) and d) are from normal pig skin.

cysts were produced within the matrix, as demonstrated in **Figure 4.1d**. These cysts consist of an inner core of debris surrounded by a ring of keratinocytes 2-10 cells thick, which appeared to be differentiated in a similar manner to the outer epithelium of mature epidermis. Keratin 10 expression was used as a marker of keratinocyte differentiation and this is clearly seen surrounding the cysts in **Figure 4.4**. In contrast, **Figure 4.5** demonstrates the smaller cysts found when Integra® was seeded with cultured cells. These cysts did not express keratin 10 and contained less debris.

Although, as mentioned above, the distribution of K14 seen in the epithelium produced by the cultured autologous keratinocytes is similar to normal pig skin **(Figure 4.3a and b)**, it is not identical. In normal pig skin the basal layers appear to have a more pronounced expression compared to the supra-basal layers. In the cultured keratinocyte samples the K14 expression is more constant throughout the epidermis.

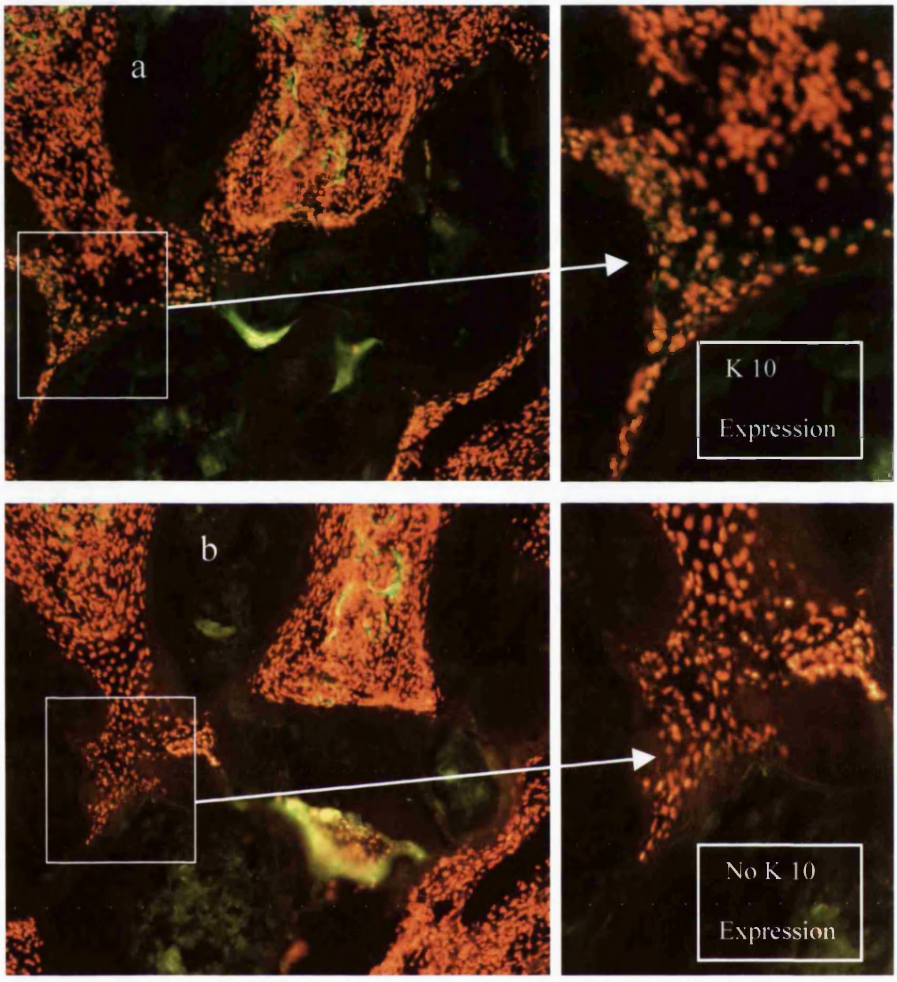


Figure 4.4 – Keratin 10 expression in keratinocyte cysts produced by non-cultured cells seeded into Integra® artificial skin. (x40 with x 100 inset)
a) The keratinocyte cysts produced by non-cultured keratinocytes consisited of an inner core of cell debris, which did not stain with propidium iodide. Differentiation was identified in keratinocytes using mouse monoclonal antibody LHP2 for keratin 10 identification. Surrounding the inner core was a ring of keratinocytes 2-10 cells thick which expressed keratin 10.
b) The negative control was performed on an adjacent slide omitting the primary antibody, but still using the secondary antibody. No fluorescence can be seen on the marginal keratinocytes in this negative control.

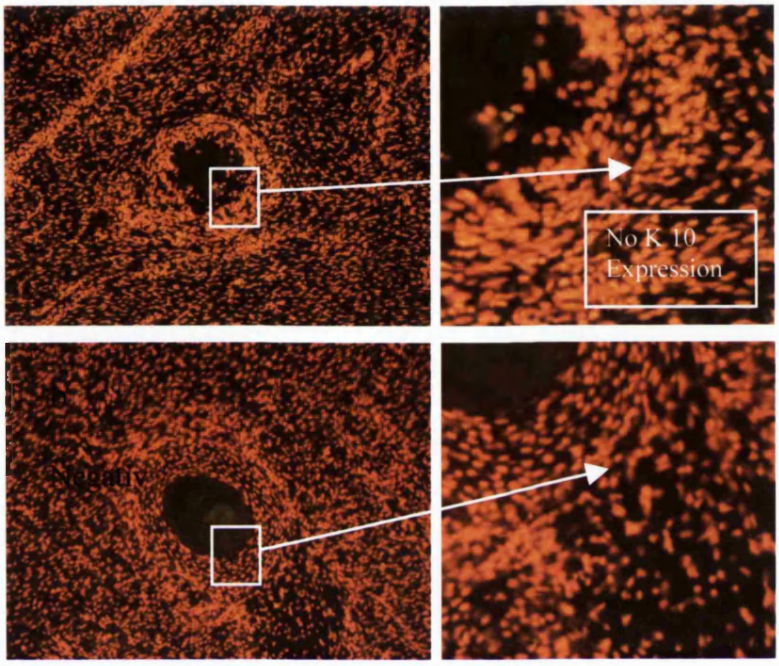


Figure 4.5 - Keratin 10 expression in keratinocyte cysts produced by cultured cells seeded into Integra® artificial skin. (x40 with x100 inset)

a) Small cysts were found in the cultured cell group. However, these cysts did not express keratin 10, as can be seen by comparison with the negative control

b) The negative control was performed on an adjacent slide omitting the primary antibody, but still using the secondary antibody. The cysts from the cultured cells also contained less debris in comparison to those from the non-cultured cells.

4.5 Discussion

The ability to use an artificial dermal scaffold seeded with freshly dispersed epithelial cells could make one-step skin reconstruction a viable clinical option. These results using **non-cultured** autologous keratinocytes to seed Integra® are in stark contrast to previous studies using **cultured** autologous keratinocytes (Kremer et al., 2000). The non-cultured cells maintain the ability to form collections of cells in the Integra® up to 1 week, but subsequently the non-cultured keratinocytes lose their mitogenic capability and differentiate by the third week. Non-commercially available collagen matrices seeded with cultured autologous keratinocytes have been shown to produce mature epithelium by three weeks (Butler et al., 1998;Compton et al., 1998). This was consistent with the result in our positive control, which used the commercially available matrix Integra®. Similarly, epithelium has been demonstrated when centrifuging non-commercially available collagen matrices with non-cultured keratinocytes, though this produced a lower percentage of epithelial cover than when using cultured keratinocytes (Butler et al., 1999). These investigators also found that there were fewer keratinocyte cysts with the use of non-cultured cells compared to cultured cells. This contrasted with our findings which showed that many well differentiated cysts formed within the Integra® seeded with non-cultured keratinocytes, and very little epithelium was produced on the wound surface.

Epidermal stem cells exhibit the greatest regenerative capacity of any basal cells. Several authors have examined the relative distribution and proportion of epidermal stem cells in normal skin. They represent a minor sub-population

(approximately 10%) of immature epidermal cells, and are quiescent at the time of isolation from the epidermis (Li et al., 1998). However, they survive and proliferate in culture, retaining their potential to differentiate and to produce neo-epidermis. Cell culture has been shown to actively select stem cells due to their greater ability to attach to cell culture substrates (Bickenbach and Chism, 1998), and it has been shown that these stem cells survive long-term when actively transplanted onto a wound (Kolodka et al., 1998). Therefore we should not be surprised that non-cultured keratinocytes behave very differently to cultured keratinocytes.

The *in vivo* success of cultured autologous keratinocytes may be due to this selection process for stem cells which is omitted when non-cultured autologous keratinocytes are used on wounds. Barrandon and Green demonstrated that a clone could be assigned to one of three classes (Barrandon and Green, 1987). The holoclone has the greatest reproductive capacity, producing colonies of which less than 5% will abort or terminally differentiate. The paraclone, on the other hand, contains only cells with a short replicative lifespan of less than 15 generations. Meroclones contain a mixture of cells of different growth potential, a transitional stage between the holoclone and the paraclone. When they are grafted to humans, keratinocyte cultures containing holoclones can regenerate epidermis that persists for years, whereas paraclones may be useful over the short term to stimulate epidermal regeneration, but are unable to contribute to long term epidermal survival. It appears that the non-cultured keratinocytes contain a high percentage of paraclones and meroclones which are capable of formation of keratinocyte collections in the first week. However, by three weeks

many of these paraclone-derived keratinocytes have differentiated, as indicated by their expression of keratin 10. The onset of differentiation appears to be associated with the loss of their ability to migrate through the Integra® matrix. This failure to reach the wound surface results in an epidermis forming within the neo-dermis.

One approach to overcome this problem may be to centrifuge the non-cultured keratinocytes towards the siliconised surface of the Integra® prior to grafting. The keratinocytes would not need to migrate through the matrix and could differentiate under the silicone with no detrimental effect to the neo-dermis. This may explain the difference in the findings of our results and other groups (Butler et al., 1999). These authors centrifuged the keratinocytes into the matrix at 50G for 15 minutes. They found a lower percentage of keratinocyte cysts. We were unable to centrifuge cells through Integra®, even with 800G for 1 hour. This may be due to differences in the pore size of this commercial product and non-commercially produced collagen glycosaminoglycan matrices.

Some success has been displayed in the use of non-cultured cell sprays used directly onto the surface of wounds (Cohen et al., 2001;Fraulin et al., 1998;Navarro et al., 2000). The difference here is that the high proportion of paraclone-derived differentiated cells will already be at the surface, and they do not need to retain their migratory capacity, as is the case with cells seeded into Integra®.

The ability to seed Integra® on the day of burn wound debridement with a suspension of non-cultured autologous keratinocytes, isolated from split skin grafts taken from non-burned areas is a very attractive proposition. This would avoid the need for a temporary wound cover. However, this study has shown that non-cultured autologous keratinocytes behave differently to cultured autologous keratinocytes in an *in vivo* wound model. Therefore, this technique is unlikely to be successful in burns patients unless we can develop a method to deliver the non-cultured cells to the siliconised surface within the Integra® matrix, without disruption of the matrix or damage to the cells from high G forces. If this can be achieved then such a one step technique may be feasible. Meshing the Integra®, perforating the silicone or matrix with laser drill holes, or perhaps attaching the matrix to the silicon with a keratinocyte fibrin glue suspension are all possibilities. This should be the aim of further investigation.

CHAPTER 5

A COMPARISON OF KERATINOCYTE CELL SPRAYS WITH AND WITHOUT FIBRIN GLUE

5.1 Introduction

The closure of large burn wounds with limited availability of autologous split skin grafts remains a challenging problem. Improvements in the survival of burns patients has increased the need for techniques which provide rapid wound closure creating minimal donor sites. In the 1950s attempts were made to seed wounds with skin particles which had been mechanically reduced into a suspension (Najarian and McCorkle, 1957;Nystrom, 1959). This produced variable results and the availability of skin graft meshing provided a more consistent alternative. Trypsinization was later used to dissociate the epidermal skin cells prior to re-seeding into wounds (Worst et al., 1982;Billingham and Reynolds, 1952). Since 1975 with the advent of a reliable method for epidermal cell culture, it has been possible to cultivate human keratinocytes *in vitro* using lethally irradiated 3T3 mouse fibroblasts in a specific culture medium (Rheinwald and Green, 1975). Surgeons can now use autologous keratinocyte sheets, which can be grown several cells thick, as a replacement for split skin autografts in major burns. However, these keratinocyte sheets have a relatively unpredictable take rate and produce a relatively unstable epidermis which is prone to blistering on minor trauma (Desai et al., 1991). Dispase®, the enzyme used to release these epidermal sheets from culture, may remove surface proteins from the keratinocytes and reduce their adhesive potential (Harris et al., 1998). This can be avoided by delivering the autologous keratinocytes to the wound surface in suspension, rather than as a coherent sheet. This has the added advantage of reducing the time needed to culture sufficient sheets to cover an adult, about 5 weeks, and the use of pre-confluent keratinocytes provides cells

which have not undergone phenotypic changes associated with contact inhibition. Therefore, keratinocytes in suspension should have a superior proliferative potential to keratinocytes in sheets.

Fraulin *et al* demonstrated the use of an aerosolised spray to deliver non-cultured epithelial cells to a wound bed in pigs (Fraulin et al., 1998). The wounds receiving the sprayed cells re-epithelialised faster than the unsprayed controls. This technique has been further developed by Navarro *et al* in pigs for use in combination with a widely meshed split thickness skin graft (Navarro et al., 2000). Sprayed non-cultured keratinocytes were applied to wounds with autologous split skin grafts meshed 3:1. The cells were suspended in growth medium and sprayed directly onto the wound in a seeding density of $2.8x10^3$ cells per cm^2. The wounds were reported as healing faster and being superior in quality when cells were sprayed. A similar technique has been used to treat hypopigmented lesions, using an aerosol spray of cultured keratinocytes and melanocytes (: Navarro et al 2000).

Fibrin glue has also been used to deliver cultured human keratinocytes to a wound in suspension. Keratinocytes can remain viable as a suspension in commercially available fibrin glue for at least five days (Jiao et al., 1998). Keratinocytes cultured on fibrin glue maintain their relative percentage of holoclones, meroclones and paraclones, demonstrating that fibrin glues do not in general induce clonal conversion and consequent loss of epidermal stem cells (Ronfard et al., 2000). Autologous keratinocytes mixed with the fibrinogen component of fibrin glue and sprayed onto leg ulcers have been demonstrated to

increase the rate of ulcer healing compared to controls (Hunyadi et al., 1988). Cultured autologous keratinocytes suspended in fibrin glue (KFGS) with allogenic skin overgraft has been used in the treatment of burns patients (Kaiser et al., 1994;Horch et al., 1994;Stark and Kaiser, 1994). The fibrin glue was used to secure the allograft to the debrided burn wound, providing a temporary wound cover, whilst allowing the keratinocyte suspension to proliferate *in vivo* and eventually replace the allograft epidermis when it underwent immunological rejection. Studies comparing standard epidermal sheet grafts with keratinocyte fibrin glue suspensions (KFGS) in a nude mouse model have demonstrated a similar rate of re-epithelialisation with either technique (Horch et al., 1998). However, reconstitution of the dermo-epidermal junction was significantly enhanced with the fibrin glue suspension technique. Autologous fibrin glue spray produced by an automated system (Vivostat™) has similarly been used to deliver sub-confluent cultured autologous keratinocytes to freshly debrided wounds in an animal model (Grant et al., 2002). The authors demonstrated that 14 days after application a 50% epithelial wound cover could be achieved on Integra® artificial skin.

Fibrin glue may have other benefits when used in the context of burn surgery. It is haemostatic, may have an antibacterial role, and it has been shown to improve the take rate of autologous split skin grafts (Currie et al., 2001). Fibrin glue is an excellent template for cellular migration which can be modified to create an optimum pore size for keratinocyte, endothelial cell and fibroblast proliferation (Pandit et al., 1998). Fibrin glue has also been demonstrated as a delivery

system for both growth factors and genetically modified cells engineered for enhanced growth factor expression (Currie et al., 2001).

Although suspensions of keratinocytes have been used both with and without fibrin glue, few comparisons have been made to demonstrate any advantage or disadvantage in the use of fibrin glue. However, Cohen et al reported that the addition of fibrin glue to an aerosol of non-cultured epidermal cells significantly enhanced the epithelialisation of wounds with unfavourable topography in a pig model (Cohen et al., 2001). In their study it was unclear what contribution was made from the improved "take" of the epidermal cell suspension or from improved migration of epidermal cells from the wound margins; although the authors comment that the healing in the fibrin-cell group was mostly from central epithelial islands.

5.2 Study objectives

We have set out to clarify the role of fibrin in epidermal cell sprays, using cultured autologous keratinocytes in a pig wound model, which incorporates polytetrafluoroethylene (PTFE) chambers around the wound margins. This prevents any contribution from the wound edges to re-epithelialisation. Therefore any difference in epithelial cover would represent an improvement in the "take" rate of the epidermal cell suspension through the addition of fibrin glue and could not be related to wound contraction or marginal regeneration.

5.3 Experimental design

A porcine model using polytetrafluoroethylene (PTFE) chambers was used for the study (cf. 2.2.15). Six full thickness wounds were made on each pig, three on either side. Three pigs were used for this study, a total of 18 wounds. Eight wounds were treated with cultured autologous keratinocytes suspended in culture medium, eight wounds were sprayed with cultured autologous keratinocytes suspended in 1ml of the fibrin/aprotinin component of Tisseel fibrin glue (Baxter, 1ml Kit: 921028) mixed with 1ml of culture medium. Wounds were allocated to treatment groups in such a way that each group had equivalent numbers of wounds in equivalent positions on consecutive animals.

5.3.1 Pig keratinocyte isolation and culture

For each pig a split thickness skin graft was harvested from the flank of the animal, and autologous keratinocytes were isolated (cf. 2.2.5) and cultured (cf. 2.2.6) through four passages over a three week period, and then harvested from culture whilst still in a pre-confluent state.

5.3.2 Cell Sprays

Pre-confluent autologous keratinocytes (fourth passage) were suspended in a concentration of $1x10^6$ per ml. Half were suspended in 2 ml aliquots in pig keratinocyte culture medium, and half were suspended in a mixture of 1ml of pig keratinocyte culture medium mixed with 1 ml of the fibrin/aprotinin component of Tisseel fibrin glue (Baxter), mixed as per manufacturers

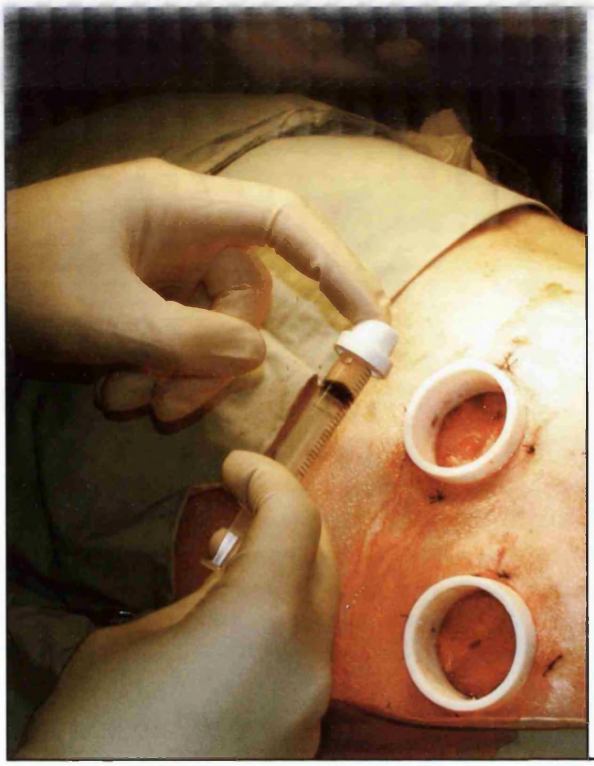


Figure 5.1 – Device used for spray delivery of keratinocyte suspensions

The PTFE wound chamber can clearly be seen around the wound margin. This prevents wound contraction and re-epithelialisation from the wound margins.

instructions. The suspensions were sprayed at a distance of 10 cm from the wounds **(Figure 5.1)**. In the latter group the thrombin component of the fibrin glue kit (500iu in 1 ml) was applied to the wound bed immediately prior to spraying. The device used for spraying the suspensions consisted of a 5 ml syringe with a spray cap supplied by Coster Aerosols Ltd, Stevenage, Herts, UK (cat no: V06222) **(Figure 5.1).** This was sterilised by steam autoclaving for 20 minutes at 121°C. A similar device has been described by Navarro *et al* (Navarro et al., 2000).

5.3.3 Wound biopsies and Histology

Whole wound excision biopsies were performed at euthanasia after the third week. The dissection was performed to include the most superficial part of the underlying muscle. Each excision biopsy was digitally photographed at a fixed distance alongside a $1cm^2$ scale. The biopsies were then cut into quadrants **(cf. Figure 3.2)** and prepared for histology by snap freezing in O.C.T. (Tissue-Tek, Miles Inc, Elkhart, IN, USA.) using liquid nitrogen. Frozen sections 15 μm thick were cut using a rocking microtome mounted inside a refrigerated insulation cabinet. Sections from each wound were stained with Haematoxylin and Eosin.

5.3.4 Immunohistochemical detection of cytokeratins and collagen

The presence of keratinocytes was confirmed using mouse monoclonal antibody LL001 (K14) supplied by Professor Irene Leigh (The Royal London Hospital). LH7.2, an anti-human collagen VII mouse monoclonal antibody which cross reacts with porcine type VII collagen, was used to confirm the location of the basal epidermal dermal junction. Frozen sections were fixed for 20 minutes in methanol and acetone (1:1), rinsed in PBS and incubated with primary antibodies for one hour. They were then incubated with the secondary antibody (sheep anti-mouse fluorescein conjugated antibody F6257, Sigma) for 45 minutes.

5.3.5 Quantification of epithelial cover

The percentage of epithelial cover was first calculated by image analysis of the digital photographs (1000x1200 pixels). The epithelial cover on each biopsy photograph was traced using the programme Image-Pro® Plus, Version 4.0 for Windows™ (Media Cybernetics, 8484 Georgia Avenue, Silver Spring, USA). The 1cm scale was also traced on each photograph, so that the epithelial cover on each biopsy could be calculated in cm^2. This technique was validated by a comparison of two independent observers. The plot of observer 1 versus observer 2 gave a straight line with R = 0.995 (not shown). Total wound biopsies contracted post-mortem, so the mean area of the wounds was calculated using the same technique.

Epithelial cover was also estimated by histogical analysis. Four sections from each wound were examined, representing two complete cross sections at 90^0 to one another. The percentage of epithelium was calculated on each slide by grid overlay, and the mean percentage of epithelial cover calculated for each wound (cf. 3.3.6).

5.4 Results

5.4.1. Controls

No epithelium was seen macroscopically or histologically in the control wounds (n=2).

5.4.2. Cell spray application

The fibrin glue suspension and the keratinocyte culture medium suspension both sprayed onto the wound surface evenly. However, the culture medium suspension dripped down the wounds to collect in a pool at the bottom of the chamber **(Figure 5.2a).** In contrast the fibrin glue suspension adhered to the wound bed with only minimal pooling **(Figure 5.2b).**

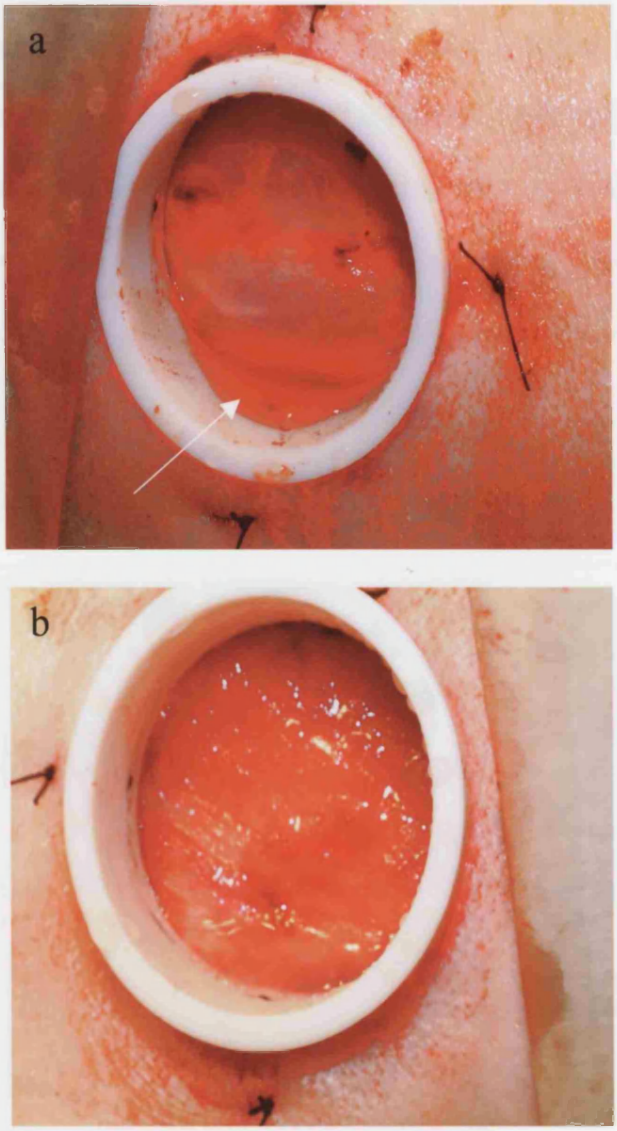


Figure 5.2 – Wounds immediately after application of cell suspensions.

a) After application of the cell suspensions to the wound bed, the culture medium cell suspension dripped down the wounds to collect in a pool at the inferior border of the PTFE chamber (arrow). b) In contrast, the fibrin glue suspension adhered to the wound bed with only minimal pooling.

5.4.3. Macroscopic wound appearance

The area of epidermal cover was variable between individual wounds (**Figure 5.3).** This may be due to the lack of dermal replacement provided by this model and the relatively low seeding density used for this study. A higher seeding density could have resulted in the wounds being completely epithelialised by three weeks which would have masked any differences between the two groups under study. The histological appearance of the wound is shown in **Figure 5.4,** which demonstrates a good histological quality stratified squamous epithelium.

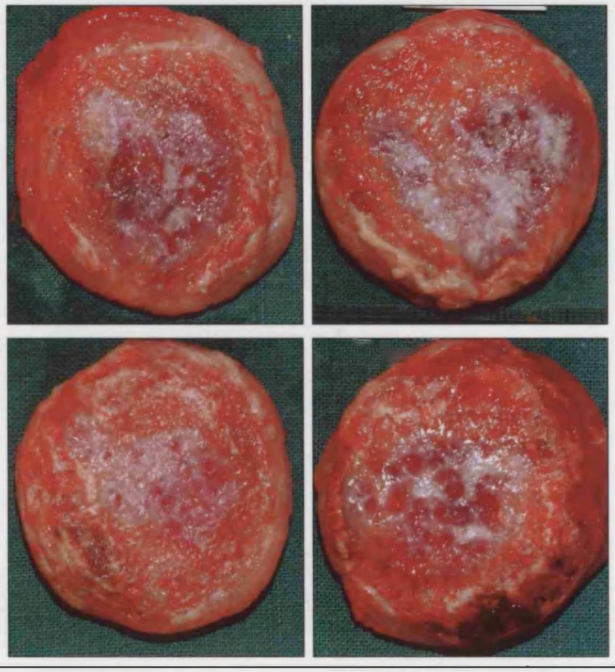


Figure 5.3

Total excision wound biopsies at 3 weeks.

There was a variable area of epidermal cover in the wounds.

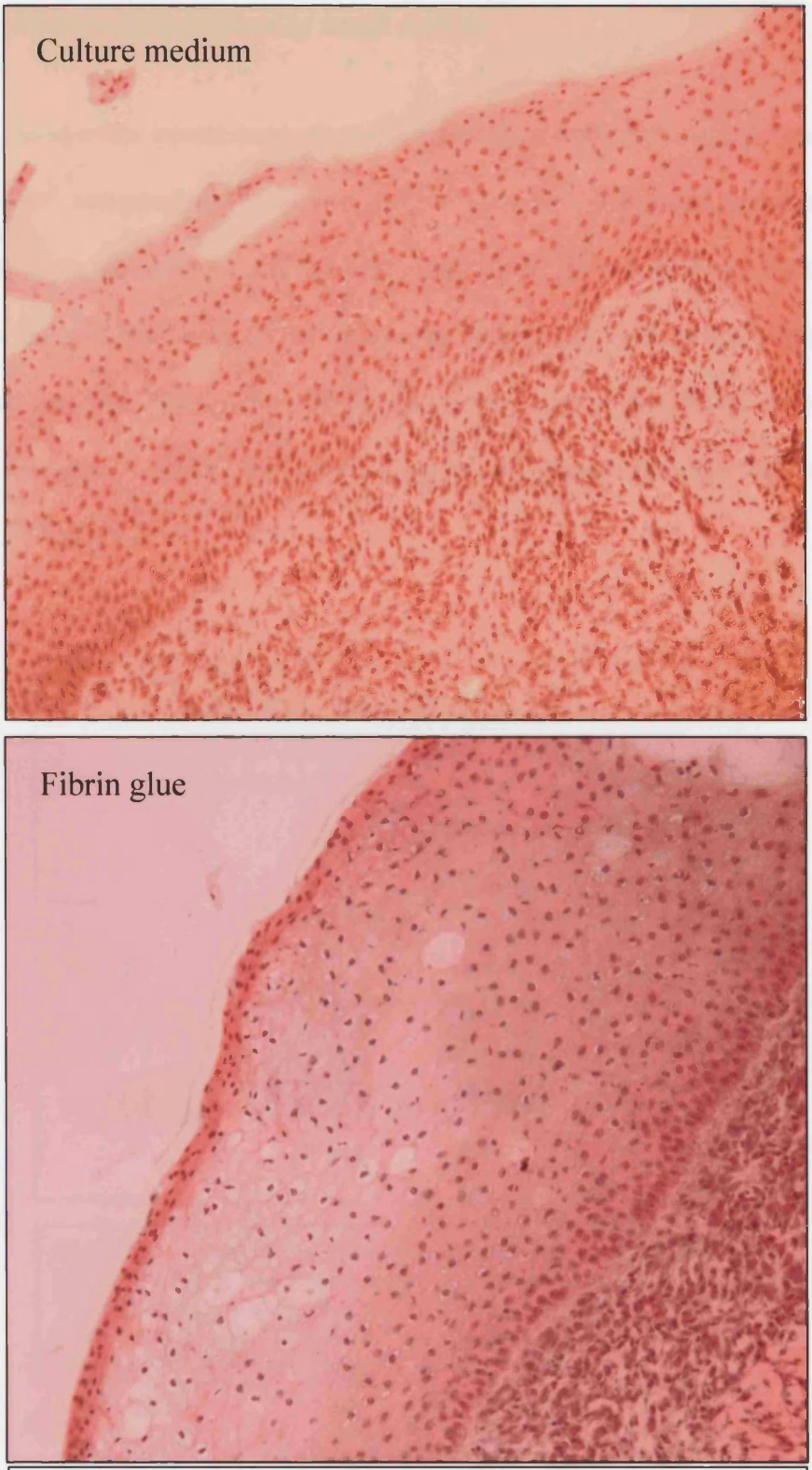


Figure 5.4 - H&E section of 3 week wound biopsies (x10)
Good quality epithelium was produced after application of keratinocytes sprayed in culture medium and fibrin glue. There was no difference on thickness or quality between the two groups.

5.4.4. Epithelialisation assessed by image analysis

Due to post-mortem wound contraction the mean of the individual wound areas was 10.1cm^2 compared to the *in vivo* area of 12.6 cm^2. The mean epithelial surface area as assessed by image analysis was 1.6 cm^2 per wound in the fibrin group (n=8), compared with 1.8 cm^2 in the non-fibrin group (n=8) **(Figure 5.5).** Statistical analysis (Sigma Stat, Jandel Scientific) with a non-paired t-test showed that there was not a statistically significant difference between the two groups (P=0.802).

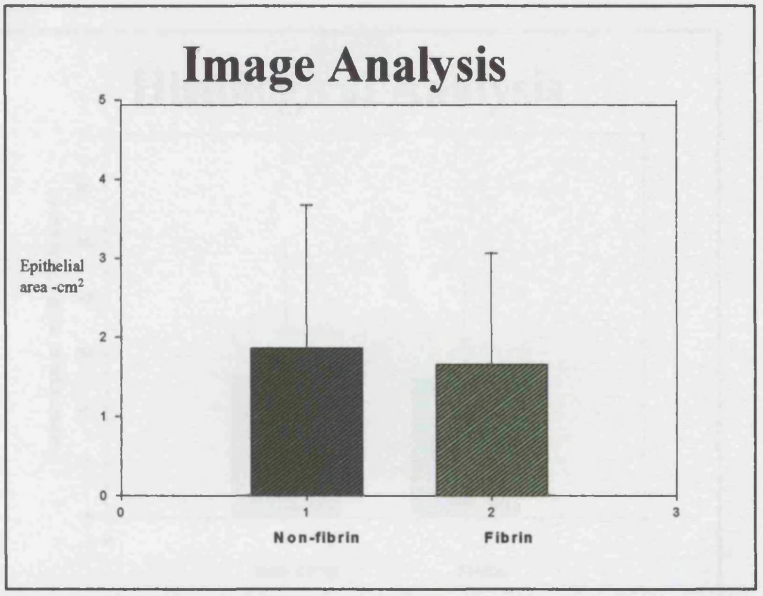


Figure 5.5 – Graph showing the epithelial area (cm^2) as assessed by image analysis in the fibrin and the non-fibrin groups.
The mean epithelial area was 1.8 cm^2 (SD 1.8) for the culture medium group and 1.6 cm^2 (SD 1.4) for the fibrin glue group. There was no statistical difference between the two groups (P=0.802).

5.4.5. Epithelialisation assessed by histological analysis

The percentage of epithelial cover as assessed by histological analysis was a mean of 25.3% in the fibrin group (n=8), compared with a mean of 25.8% in the non-fibrin group (n=8) **(Figure 5.6)**. The data failed a normality test so a Mann-Whitney Rank Sum Test was performed. The fibrin group had a median of 29.4% (25% - 4.4, 75% - 49.4), whilst the non-fibrin group had a median of 18.8% (25% - 5.6, 75% - 42.5). There was not a statistically significant difference between the two groups (P=0.878).

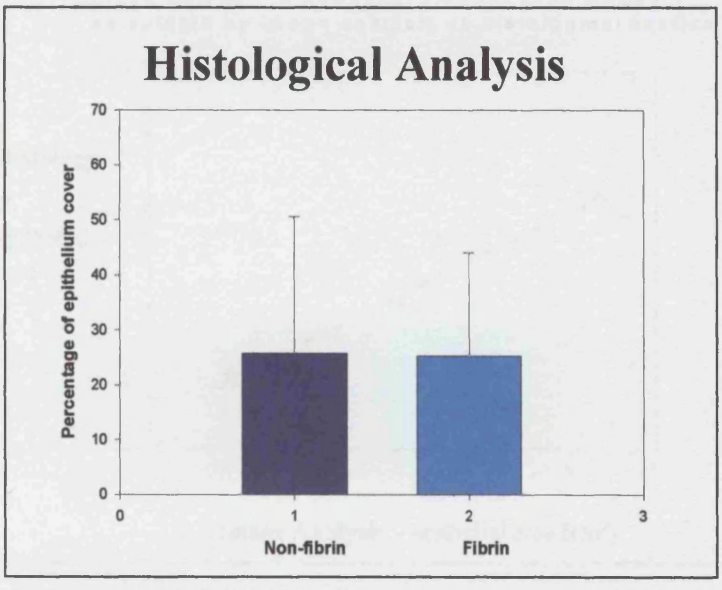


Figure 5.6 - Graph showing epithelial area as a percentage of total wound area in the fibrin and the non-fibrin groups following histological analysis.
The mean percentage of epithelium in the fibrin group was 25.3%, compared with a mean percentage of epithelium in the culture medium group of 25.8%. There was not a statistically significant difference between the two groups (Mann-Whitney Rank Sum Test P=0.878).

5.4.6. Comparison of image analysis with histological analysis

For each wound the epithelial area as calculated by image analysis was compared to the epithelial percentage as calculated by histology. A regression analysis was then performed to compare the accuracy of the two methods **(Figure 5.7)**. This gave an R value of 0.967 demonstrating a very good correlation.

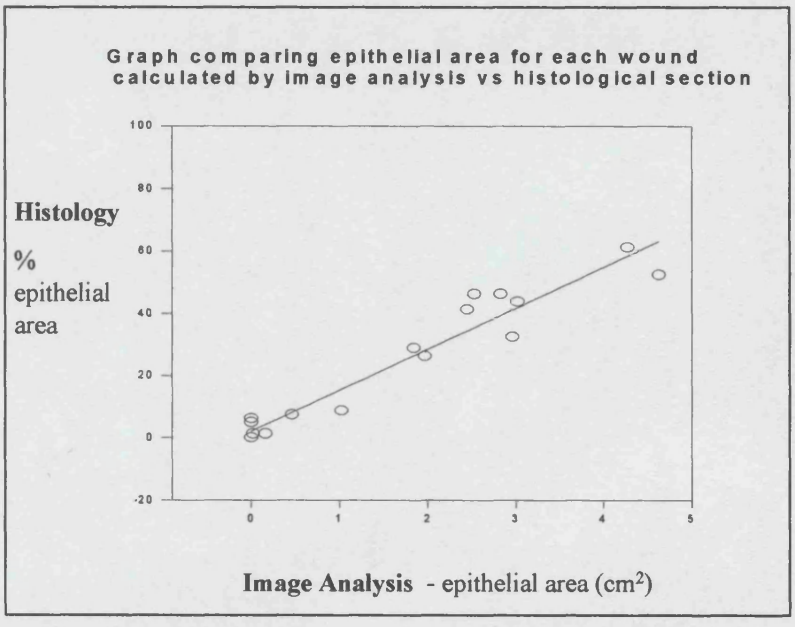


Figure 5.7 – Regression analysis comparing the epithelial area for each wound assessed by image analysis with the percentage epithelial cover for the same wound assessed by histological analysis (R=0.967).

5.4.7. Immunohistochemistry

Immunohistochemistry **(Figure 5.8)** showed slight difference in the Keratin 14 expression in either group. The fibrin group had a more pronounced K14 expression throughout the epidermis. In normal pig skin the basal layers appear to have a more pronounced expression compared to the supra-basal layers. In the cultured keratinocyte samples the K14 expression is more constant throughout the epidermis. Collagen VII was present at the basal epidermal dermal junction in both groups **(Figure 5.9)**.

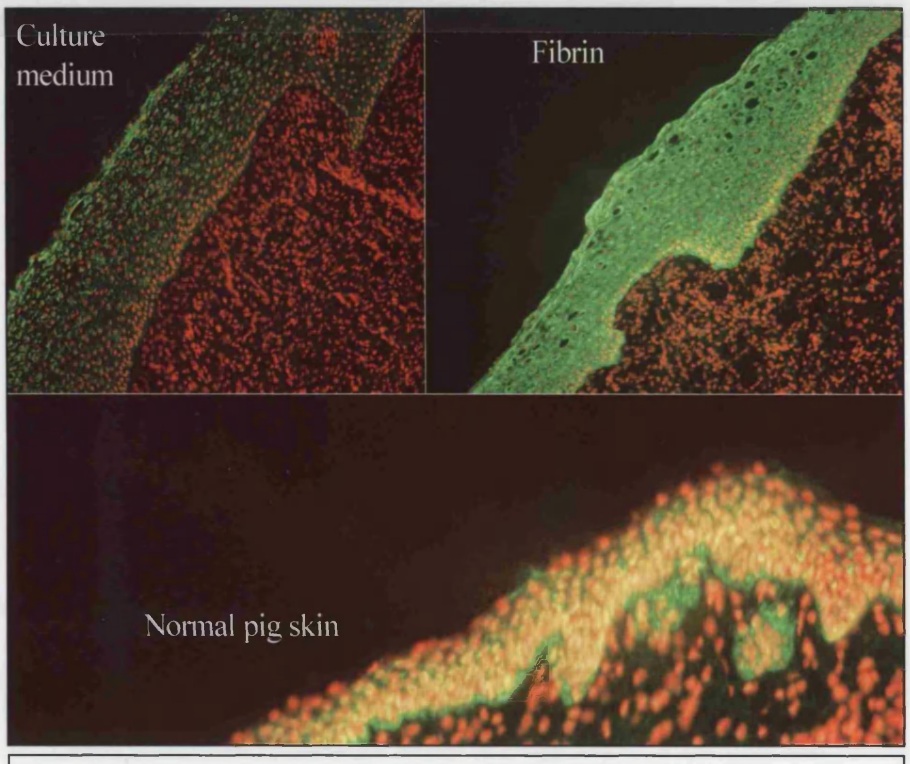


Figure 5.8 – K14 immunohistochemistry of epithelium produced by cultured autologous keratinocytes sprayed onto full thickness wounds
There is no difference between the two experimental groups.

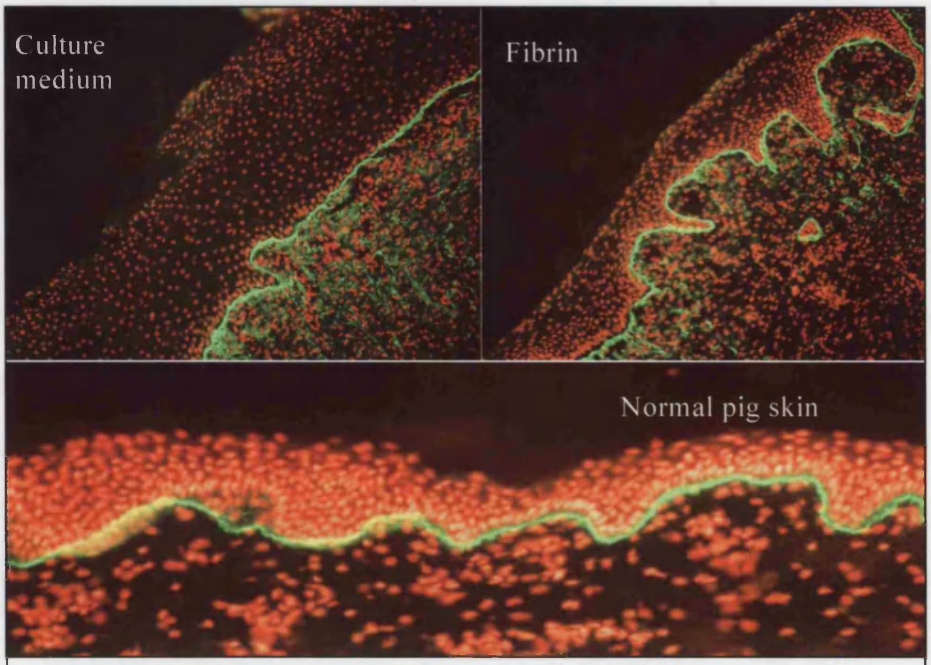


Figure 5.9 – Collagen VII immunohistochemistry of epithelium produced by cultured autologous keratinocytes sprayed onto full thickness wounds
Both groups demonstrate increased cellularity of the dermis compared with normal pig skin. Collagen VII was expressed at the dermo-epidermal junction of both groups by the third week. There is also enhanced collagen VII expression within the dermis, which probably represents small blood vessels.

5.5 Discussion

Previous studies, outlined in the introduction, suggest that fibrin glue may have a beneficial effect when used to deliver cell suspensions to a wound bed. When cell suspensions in culture medium alone are applied to a vertical wound bed, the suspension can often be seen to drip down the wound face and pool in its inferior aspect, apparently wasting many of the applied cells. Cell suspensions in fibrin glue adhere to the wound bed immediately, as was observed in this study. This could be anticipated to result in a faster rate of wound re-epithelialisation. However, our results suggest that fibrin glue has no beneficial

effect on epidermal cell suspension "take" rates. When a suspension of 2 million cultured autologous keratinocytes was sprayed onto 12.6 cm^2 wounds, there was no statistical difference in the percentage of epithelium produced at three weeks if fibrin glue was used, compared to sprayed cells suspended in culture medium alone. Both with and without fibrin approximately 25% of the 12.6 cm^2 wound area was epithelialised after 3 weeks, a surprising finding.

Fibrin glue prevents cells from dripping out of the wound. However, it will also hold a proportion of cells away from the wound bed. Transplanted epidermal cells initially survive by serum imbibition (plasmatic circulation) with subsequent in-growth of vascular buds (neovascularisation). It is possible that the fibrin glue prevents this process. A greater percentage of the transplanted epidermal cells may die due to their increased distance from the wound bed, before new vessel formation is achieved. Studies have shown that fibrin glue is non-toxic to cultured autologous keratinocytes (Ronfard et al., 2000)and in fact it is a good medium for both culture (Ronfard et al., 1991) and delivery (Hunyadi et al., 1988;Horch et al., 1998) of keratinocytes. However, these studies have all compared the delivery of keratinocyte suspensions in fibrin glue with a control consisting of either fibrin glue only (i.e. no keratinocytes) or no treatment (i.e. no fibrin glue and no keratinocytes). They do not compare wounds treated with keratinocytes either with or without fibrin glue as we have done here. The potential benefit of fibrin glue in this context has been "assumed".

Cohen *et al* examined the benefits of fibrin glue in aerosolised form for the delivery of non-cultured epidermal cells into contracting pig wounds (Cohen et al., 2001). In contrast to our findings these authors found a beneficial effect in the use of fibrin glue. Those authors found that wounds treated with fibrin glue and cells initially contracted at a slower rate, and that wounds treated with fibrin glue and cells re-epithelialised twice as quickly. One difference between the two studies is that the other groups looks at contracting wounds, whereas in our study the PTFE wound chambers prevent both wound contraction and migration of keratinocytes from the wound margins. Wound keratinocytes, in contrast to non-wound keratinocytes, express functionally active integrin receptors for fibronectin (Toda et al., 1987), a constituent of fibrin glue. This has been shown to facilitate their migration across this matrix (Clark, 1990). Fibronectin has also been shown to increase the rate of keratinocyte spreading and replication (Weiss et al., 1998). Therefore, it is entirely possible that the difference in epithelial cover seen in their study is due to the beneficial effect of fibrin glue on keratinocyte migration from the wound edges and not due to the non-cultured keratinocytes delivered to the wound bed in the fibrin glue. This may explain the contrasting results.

Cohen *et al* used non-cultured keratinocytes, in contrast to the cultured keratinocytes used in our study. Cell culture has been shown to actively select holoclones (Barrandon and Green, 1987), epidermal cells with high proliferative potential, due to their greater ability to attach to cell culture substrates (Bickenbach and Chism, 1998), and it has been shown that these holoclones survive long-term when actively transplanted onto a wound.(Kolodka et al.,

1998) Non-cultured cell suspensions on the other hand will have a high percentage of paraclones, cells with a lower proliferative potential, and differentiated cells. These paraclones will have a short replicative lifespan (Barrandon and Green, 1987). Therefore, the non-cultured cells delivered to the wound in suspension will have only a small number of proliferating cells in comparison to the wound margins. This again suggests that this is the site at which fibrin glue was providing its beneficial effect.

Fibrin glue costs around £300 for 5 ml in the UK. Cell suspensions are becoming an increasingly popular method of delivery of cultured keratinocytes to the wound bed in burns patients. If this trend continues, then this study has significant financial implications. However, we have not examined other potential benefits of fibrin glue such as haemostasis, or protection from dressings. This may be an alternate and highly justifiable reason for adding fibrin glue to cell suspensions of keratinocytes used in burns patients. This study has shown that there is neither benefit or disadvantage in the isolated environment of rigid PTFE chambers in the pig. Cell suspensions have also been used extensively with widely meshed autologous split skin grafts (Navarro et al., 2000;Horch et al., 1994). In this case the fibrin glue may also improve the migration of the keratinocytes from the autologous split skin graft margins.

Pooled blood products obviously carry a risk of disease transmission, particularly Hepatitis B and more recently HIV. Donor screening, heat-treating and the use of a solvent/detergent suspension for inactivation of lipid-enveloped blood-borne viruses in plasma derivatives (Horowitz et al., 1988) has made

these products safer. However, a theoretical risk of disease transmission still exists and fibrin glue should only be used where a clinical benefit has been demonstrated.

In this study we have used a relatively low concentration of cells ($2x10^5$ cm^2). It is conceivable that a higher concentration of cells may improve the results when using fibrin glue. In a high concentration suspension, a greater number of cells may be held close to the wound bed, enhancing survival. However, a higher concentration of cells in a smaller volume of solution would also reduce the fluid "dripping off" the wounds, enhancing survival for the non- fibrin group. Clearly this is an area for further studies.

This study has demonstrated that cultured autologous keratinocytes can be delivered to a full thickness wound bed with the use of a simple syringe and spray head. The keratinocytes sprayed in a relatively low concentration of 1 million cells per ml produced a good quality epithelium over 25% of the wound by three weeks. The PTFE chambers prevented any re-epithelialisation from the wound margins, so this epithelium must have originated from the keratinocyte suspensions sprayed onto the wounds. Fibrin glue suspensions were not found to have any advantage in terms of percentage of epithelial cover produced at three weeks when compared to suspensions in culture medium alone. This contradicts some previous studies and further investigation is clearly needed to identify optimal cell density and liquid volumes for spray re-epithelialisation.

CHAPTER 6

AN INVESTIGATION INTO FIBROBLAST SURVIVAL IN INTEGRA® USING *LACZ* LABELLED AUTOLOGOUS AND ALLOGENIC FIBROBLASTS

6.1 Introduction

Rheinwald and Green recognised that human keratinocytes would only grow *in vitro* in the presence of fibroblasts (Rheinwald and Green, 1975). This is thought to be due to interactions between the two cell types, such as the production of stimulatory cytokines and growth factors. Monocultures of normal human keratinocytes or fibroblasts express very low levels of collagen VII, the major structural protein of the anchoring fibrils in the basement membrane. However, collagen VII expression is greatly stimulated when fibroblasts and keratinocytes are co-cultured (Konig and Bruckner-Tuderman, 1991). This suggests that epithelial-mesenchymal interactions are necessary for efficient synthesis of collagen VII and formation of basement membrane anchoring fibrils.

Several authors have compared composite skin equivalents with and without fibroblasts. It would seem that fibroblasts play an important role in the *in vitro* formation of these composites. Maruguchi (Maruguchi et al., 1994) compared the *in vitro* proliferation of keratinocytes on a collagen sponge with and without fibroblasts seeded into the uppermost layer of the sponge. They demonstrated that keratinocytes proliferated and differentiated appropriately only in the collagen matrix which included fibroblasts. Similarly, the keratinocyte seeding efficacy of hyalauronic acid membranes has been shown to improve from 75% to 95% when proliferative allogenic fibroblasts were grown on the membranes as a feeder layer (Lam et al., 1999).

Human skin constructs have been grafted into nude mouse models, with and without human fibroblasts. When human fibroblasts are used as part of the composite, human type I collagen and elastin fibers can be detected by species specific antibody labelling (Demarchez et al., 1992). However, when human fibroblasts are not incorporated into the complex, the composite is invaded by mouse fibroblasts with a subsequent increase in the production of murine collagen. However, if the dermal-epidermal junction of a composite can be either preserved (as with some methods of allodermis preparation) or formed *in vitro* by co-culture with xenogenic or allogenic fibroblasts, then it may not be essential to deliver autologous fibroblasts to the wound in a composite (Chakrabarty et al., 1999). Cui *et al* have demonstrated that autologous keratinocytes pre-cultured on allodermis produce a better, more durable epithelial cover than autologous keratinocytes alone (Cui et al., 1999). Other authors have tried to produce a complex using autologous fibroblasts seeded into a collagen lattice and then co-culturing autologous keratinocytes onto this dermal equivalent (Coulomb et al., 1998). However, the authors reported poor cosmetic results, and changed the technique to a two-step grafting procedure. Despite this a number of techniques have now been developed which create complex skin substitutes from both autologous keratinocytes and autologous fibroblasts (Boyce, 1998;Boyce et al., 1999;Caruso et al., 1999).

Several studies have attempted to trace the fate of transplanted allogenic fibroblasts, though few have followed that of autologous fibroblasts. Sex-mismatched allogenic fibroblasts seeded into type I collagen gels and overlain with epidermal keratinocytes were shown to be present in human wounds 2.5

years after transplantation (Otto et al., 1995). This was done by in situ hybridisation of the PHY2.1 repetitive Y chromosome sequence. Similarly, transplanted allogenic rabbit fibroblasts have been labelled with a fluorescent vital dye, CM-DiI, and injected intradermally into the dorsal skin of adult rabbits (Hebda and Dohar, 1999). Despite a gradual reduction in the cell density, cells were still detectable by 56 days into the study. In contrast, Kolokol *et al* found that cultured allogenic fibroblasts labelled with fluorescent acridine orange which were transplanted onto a burn wound were destroyed within two days (Kolokol'chikova et al., 2001).

Virally transduced allogenic fibroblasts have been transplanted into the dermis of immunocompetant and immunodeficient (SCID) mice to assess donor cell survival (Radfar et al., 2000). The fibroblasts carried the *LacZ* marker. They were injected into the dorsal skin of adult mice, and skin biopsies were assayed for *LacZ* expression. Immediately after transplant there was a transient decrease in cell numbers. However, viable allogenic fibroblasts were present in appreciable numbers up until the end of the study period at 7 days. Moreover, the survival of the transplanted cells was similar in both the immunocompetant and immunocompromised groups, and there was no evidence of acute inflammation or rejection in either group.

Several commercial skin replacements contain allogenic fibroblasts (Apligraf®, Dermagraft®, and Transcyte®). It is believed that they function by producing exogenous growth factors which promote wound healing, however, the long term survival of the fibroblasts remains a subject of debate.

In this study, performed in conjunction with Dawn Mann (Blond McIndoe Centre, East Grinstead), we aimed to investigate the fate of both autologous and allogenic fibroblasts, delivered to the wound bed seeded into Integra®, using *lacZnls* gene marking techniques in an *in vivo* wound model.

6.3 Experimental design

In this study three pigs were used with a total of 18 wounds. 7 wounds were grafted with Integra® seeded with autologous fibroblasts labelled with *lacZnls*, 7 wounds were grafted with Integra® seeded with allogenic fibroblasts labelled with *lacZnls* and 4 wounds were grafted with Integra® only as a control. These wounds were distributed between the pigs in such a way that each pig had a mixture of autologous, allogenic and control wounds. After 10 days the silicone from the Integra® was removed and a thin split thickness skin graft (200μm thick) was applied to each wound.

6.3.1 Fibroblast transduction

Porcine fibroblasts were isolated from split thickness skin grafts as previously described (cf. 2.2.9), and cultured for 6 days prior to being exposed to the *MFGlacZnls* retrovirus over a subsequent period of 5 days. The fibroblasts were labelled with the *lacZnls* reporter gene using supernatant transduction with the *MFGlacZnls* virus produced from a PT67 cell line (cf. 2.2.10).

Flasks of transduced fibroblasts were stained for β-galactosidase activity using the X-gal substrate (cf. 2.2.25) to determine the transduction frequency of the *lacZnls* labelled (cf. 2.2.11). The transduction frequency was 34.6% **(Figure 6.1a)**. Cells grown for a further 6 weeks in culture continued to express *lacZnls* in 27% of cells **(Figure 6.1b)**.

6.3.2 Integra® graft preparation

A seeding density of $5x10^5$ per cm^2 was used. Each wound measured 12.5 cm^2

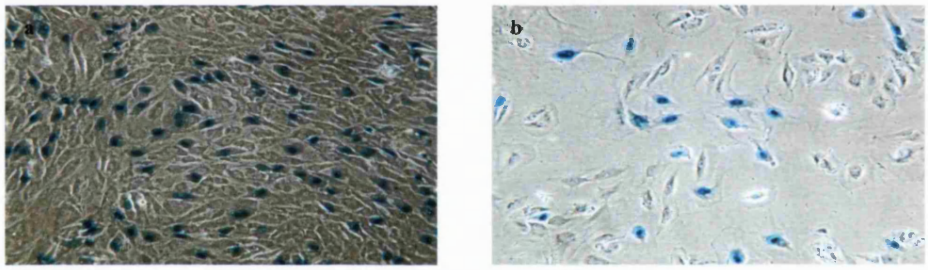


Figure 6.1 – Pig fibroblast transduction rates

a) Transduction rate of 34.6% in pig fibroblasts primary cultures *in vitro.*
b) Transduction rates of 27% in pig fibroblasts after 6 weeks of further culture.

so a total of $6.25x10^6$ fibroblasts were applied to each wound. This was applied to the non-siliconised surface of the Integra® after it had been washed three times in saline and then patted dry with a gauze swab. The cells were delivered to the Integra® in 2 mls of fibroblast culture medium. The seeded Integra® was sutured to the wound bed and (cf. 2.2.15-16). One specimen was frozen immediately in OCT for cryosection. The number of fibroblasts in a $200\mu m^2$

area of this graft was counted in a 15μm section for comparison with similar thickness sections of the wound biopsies.

Total wound excision biopsies were taken at 1,2,3 and 4 weeks. The specimens were divided into quadrants and frozen for cryosection into 15μm sections. The slides were then stained with H&E and X-gal (cf. 2.2.25).

6.4 Results

The Integra® seeded with both autologous and allogenic fibroblasts were well incorporated into the wounds. Histology of these wound biopsies demonstrated a rapid fibroblast migration and proliferation from the underlying tissue, as was seen with non-seeded Integra® (cf. 3.4.3.1.).

Fibroblasts labelled with *LacZnls* were detected in wounds grafted with Integra® seeded with both **autologous and allogenic** fibroblasts at 9 days **(Figures 6.2, 6.3).** There were fewer allogenic fibroblasts seen than autologous fibroblasts, despite identical seeding densities. Similar numbers of *LacZnls* labelled fibroblasts were detected at 2 weeks in wounds grafted with **autologous** fibroblasts as had been seen at 9 days. Wounds grafted with **allogenic** fibroblasts also contained some cells staining positively with X-gal at two weeks, but cell numbers were very low. It was not possible to see any labelled **autologous** or **allogenic** fibroblasts in the wound biopsies at 3 or 4 weeks.

Fibroblast counts were taken from images representing a $200\mu m^2$ area of one of the wound biopsies at one week, and from images representing a $200\mu m^2$ area of a section of seeded Integra® prior to implantation into the wound. These show that there are 100 fold more fibroblasts present in the biointegrated Integra® **(584 in $200\mu m^2$)** than the number of labelled fibroblasts originally seeded into a sheet of Integra® at 5×10^5 per cm^2 **(6 in $200\mu m^2$) (Figure 6.4**).

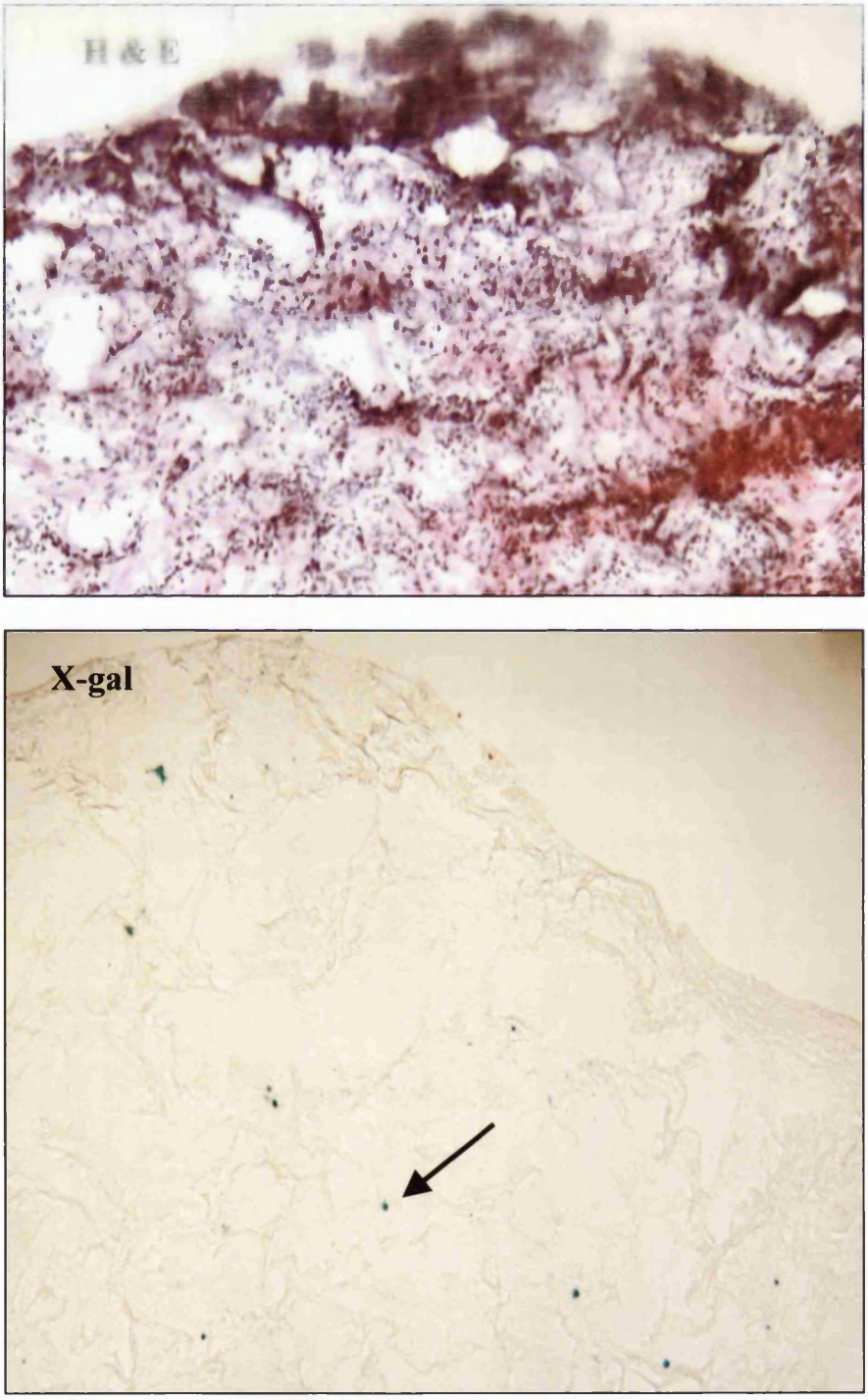
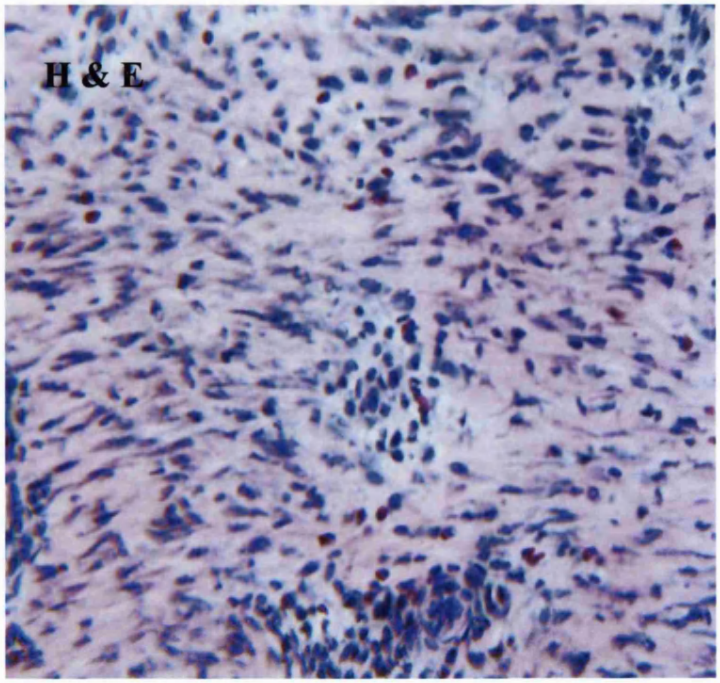


Figure 6.2 - Autologous *lacZnls* positive cells within Integra® neodermis at 9 days (x40).
The two pictures represent adjacent sections, one stained with H&E and one with |X-gal, both at the same magnification. The autologous fibroblasts which retain the reporter gene are clearly seen as blue spots (arrow) with the X-gal stain. The transduction rate was 34.6 %, so the wound will in fact contain more than twice this number of the autologous fibroblasts seeded into the matrix.



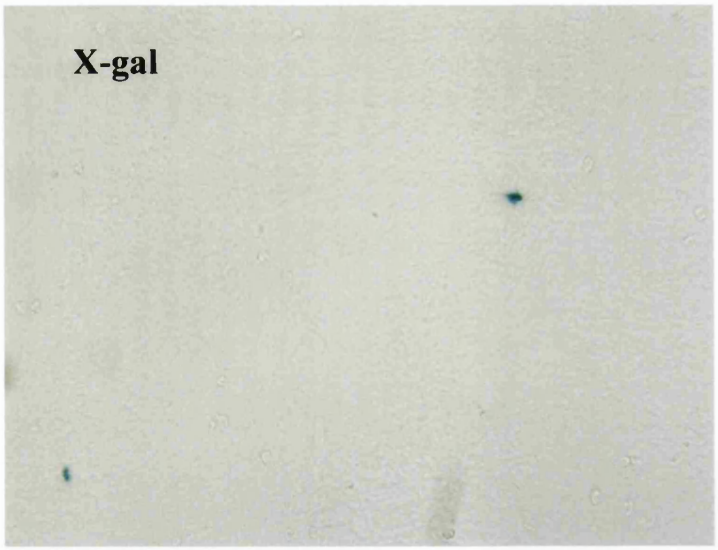


Figure 6.3 - Allogeneic *lacZnls* positive cells within Integra® neodermis at 9 days (x100)**.**
The two pictures represent adjacent sections, one stained with H&E and one with X-gal, both at the same magnification. The autologous fibroblasts which retain the reporter gene are clearly seen as blue spots with X-gal stain. There were fewer allogenic fibroblasts seen in the wound biopsies at 9 days for a given area than autologous fibroblasts (cf. figure 6.2)

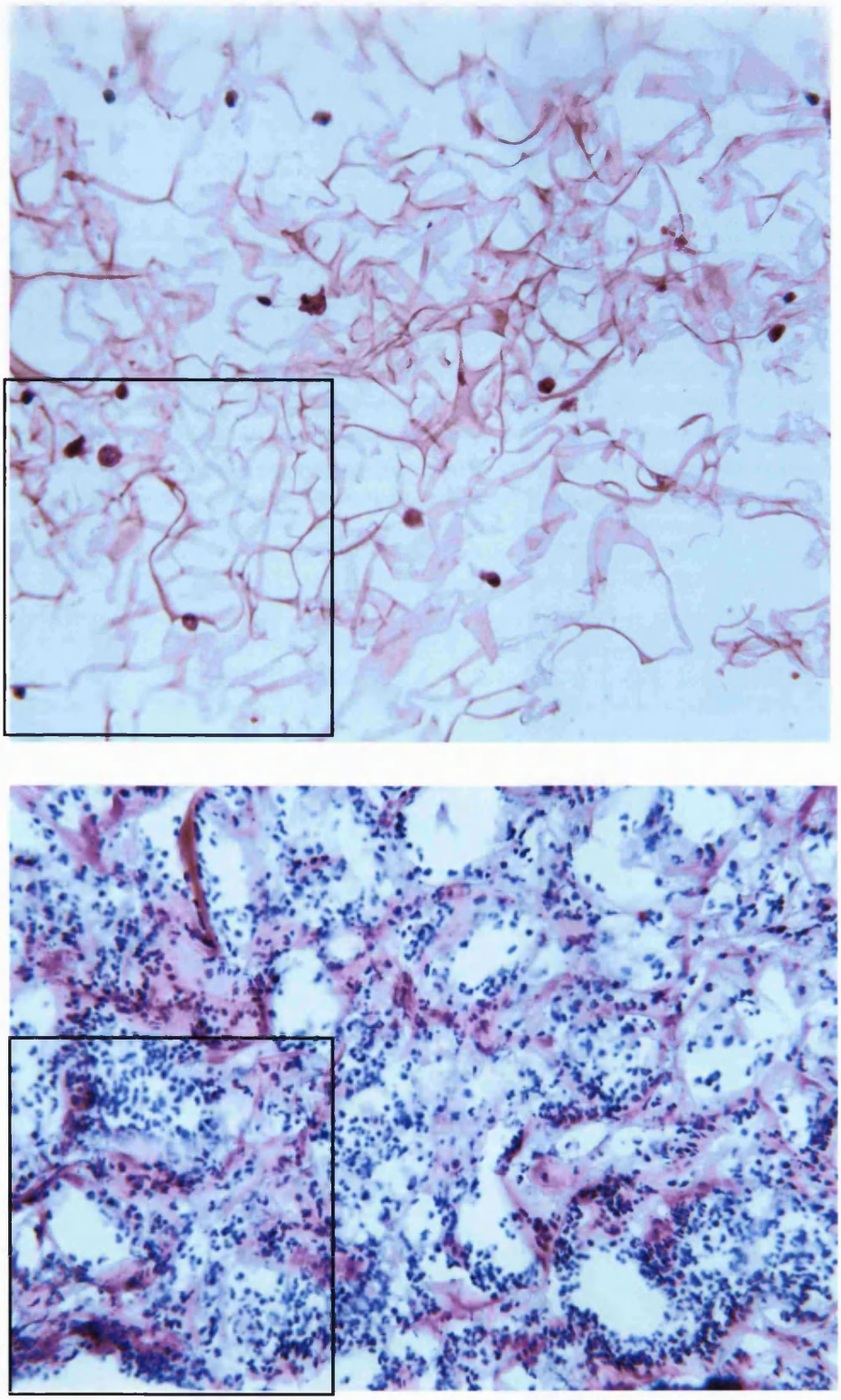


Figure 6.4 – Comparison of fibroblast numbers in Integra® seeded in vitro with Integra® at nine days in vivo.

a) Integra® seeded with 5 x 10^5 fibroblast per cm^2 prior to grafting into the wound bed. The fibroblast count was 6 in 200μm^2 (marked area).

b) Integra® nine days after grafting into pig wound. The fibroblast count was 584 in 200μm^2 (marked area) .

6.5 Discussion

Autologous and allogeneic *LacZnls* labelled fibroblasts can be detected in the porcine wound healing model up to 2 weeks. This result is similar to those found by other authors (Radfar et al., 2000). No labelled fibroblasts could be detected at three or four weeks. However, we have shown that *in vitro* fibroblasts expressed *lacZnls* for at least 10 weeks. What is the fate of the labelled cells after this two-week period?

There are several possibilities. Firstly, that the labelled fibroblasts are simply not present in large enough quantities to be seen. Secondly, the labelled fibroblasts are being recognised as "foreign" by the immunocompetant host and destroyed by cell mediated immune systems. Thirdly, the labelled fibroblasts may die prematurely as a consequence of the change in their DNA subsequent to retroviral transduction. The ability of the labelled cells to survive *in vitro* makes the third hypothesis less likely. The autologous cells were found in greater numbers than the allogenic cells, despite similar seeding densities. This may favour the second hypothesis as a likely cause. However, the sparsity of the labelled cells at three weeks may be a combination of the above factors.

When we examined the number of fibroblasts in a $200\mu m^2$ area of a $15\mu m$ section, we found a 100 fold difference in the pre-grafting density of fibroblasts when compared to the 9 day post-grafting density. These fibroblasts are derived from local mesenchymal cells, particularly those associated with blood vessel adventitia (Ross et al., 1970). The rapid expansion is due to a combination of migration and proliferation, induced by cytokines produced from platelets,

macrophages and lymphocytes. In the first 24 hours, migration appears to be the most important factor. Irradiated fibroblasts have been shown to invade a fibrin gel almost as well as non-irradiated cells, indicating that migration is important in this process which is not proliferation dependant (Greiling and Clark, 1997). The mitotic indices in granulation tissue have been studied in a rat model (Spyrou et al., 1998). The investigators demonstrated a high mitotic rate in the surrounding fascia and dermal tissue in the first few days after wounding. No increase in the mitotic rate of the granulation tissue fibroblasts was seen until day 3 after wounding, with a return to day 1 levels by the fifth day.

Schreier *et al* examined the relative contributions of migration and proliferation in a wound healing model *in vitro* using mitomycin C to inhibit proliferation (Schreier et al., 1993). They found migration to account for 40-87% of the fibroblast contribution, depending on the growth factor studied. Their control, with no added growth factor, showed 62% of the fibroblasts were derived from migration rather than proliferation. Similarly, keratinocytes have been demonstrated to double the mitotic rate of their basal epithelium as a response to injury (Bos and Burkhardt, 1981). However the same study demonstrated that non-epithelial cells apparently recruit mainly from migrating cells into the epithelium, while proliferation in situ plays a secondary role. Therefore, it would seem that over half of the fibroblasts seen in a wound are derived from a rapid migration from the underlying tissue within the first 48 hours.

The mean cell cycle length in isolated fibroblasts has been estimated as 9-15 hours (Kasinathan et al., 2001;Spyrou et al., 1998). This correlates well with the

personal observation that a confluent flask of 3T3 fibroblasts will reach confluence in secondary passages in about four days if split into five flasks. In our model, we estimated that 20 fibroblasts migrated into our 200μm^2 area of a section in the first 48 hrs, compared to the 6 labelled fibroblasts already in the Integra®. In the next seven days these would progress through a further 11 cell cycles, producing potential $2x10^{14}$ fibroblasts. The six labelled fibroblasts seeded into the Integra® at time zero would have the potential for 14 cell cycles, but this would still only be a total of $4.7x10^{11}$ labelled fibroblasts. In reality other factors prevent such huge numbers of fibroblasts being produced in such a small volume of tissue. However, this model demonstrates how the ratio of labelled to unlabelled fibroblasts can become disproportionate after this initial migration.

We have used a relatively low seeding density here and two further studies would be worthwhile. Firstly, to repeat the experiment with a higher seeding density of fibroblasts. This may prevent the labelled cells from being "swamped" by migrating fibroblasts from the surrounding tissue. Secondly, to perform a labelled fibroblast longevity study in an immunocompromised animal model. This would elucidate any reduced proliferative capacity of the labelled cells, without the possibility of the host recognising the label as a foreign protein and destroying it.

CHAPTER 7

WOUND CONTRACTION RATES

7.1 Introduction

Wound contraction can be beneficial in the closure of large cutaneous defects, but scar contracture is often detrimental. Large defects will close by contraction alone, provided the wound is maintained clean and infection free, and the patient is maintained in positive nitrogen balance. However, the cosmetic and functional end result is often sub-optimal. The development of hypertrophic scars and subsequent scar contracture has been shown to be more common in wounds which are closed with a time delay (Deitch et al., 1983). This is a difficult problem for burns surgeons. Patients with major burns involving greater than 50% of their total body surface area will have inadequate donor sites to harvest split skin grafts in order to achieve rapid closure. Therefore, these patients are prone to the development of hypertrophic scars and scar contractures. The use of synthetic materials which offer rapid wound closure has now been used extensively in burns patients (Chou et al., 2001;Dantzer and Braye, 2001). A hierarchy exists for the relative prevention of wound contraction by split skin grafts, full thickness skin grafts and flaps (Sawhney and Monga, 1970;Corps, 1969), but it is not clear exactly how artificial skin replacements compare in their ability to prevent a wound from contracting.

Humans are unique in being the only animal to have hypertrophic and keloid scarring. However, wound contraction occurs in most animals. Earlier studies have shown that the rate of wound contraction is species specific, but in each species the change in wound area with time due to contraction was found to be consistent with a constant linear rate of movement of the wound margins

The role of the epidermis in wound contraction and hypertrophic scar formation is not clear. Activated keratinocytes (expressing the hyper-proliferative keratins K6 and K16) have been shown to be present in hypertrophic scars throughout the basal and supra-basal layers (Machesney *et al*, 1998). In contrast, they are only found in the basal layer of normal skin and non-hypertrophic wounds. These activated keratinocytes are a source of many cytokines, suggesting that wound contraction and hypertrophic scar formation may not be an isolated dermal phenomenon.

(Snowden, 1981). Wound contraction may be inhibited by skin grafts due to their stimulation of apoptosis of myofibroblasts (see Chapter 1 section 1.9). If this is the case, and hypertrophic scar formation is a consequence of myofibroblast survival and over-production of collagen, then wounds which contract less are unlikely to form hypertrophic scars. This correlates well with the clinical observation that full thickness skin grafts produce less hypertrophic scarring than split thickness skin grafts. Therefore, if an artificial skin replacement can be shown to produce less wound contraction in an animal model, it is very likely that the same product will produce less hypertrophic scar formation in humans.

7.2 Study objectives

We have investigated wound contraction using Integra® artificial skin in a pig wound model. Contraction of full thickness skin wounds dressed with a simple paraffin gauze dressing were compared with wounds grafted with split thickness skin grafts and wounds grafted with Integra®. The wounds grafted with Integra® were subsequently grafted with an ultra thin split thickness skin graft as normal clinical practice for this material.

7.3 Pig wound contraction model

Three pigs were used for this pilot study. Three circular wounds were created on both flanks of each animal, 4cm in diameter, with an area of 12.6cm^2. This gave a total of 18 full thickness wounds. Each wound was either left open or grafted with a split thickness skin graft 250μm thick (cf. 2.2.14) or with

Integra®. The wounds were dressed every third day with Jelonet® and gauze and a protective jacket (Chapter 2, figure 2.4). When the Integra® was fully bio-integrated the silicone layer was removed and an ultra thin split skin graft (150μm) was applied to the wound surface, this was usually at ten days. The wounds were distributed on the animals so that the same number of each wound type was on each wound position. This eradicated any bias due to different contraction rates in different wound positions.

The wounds were photographed against a scale with a Fuji coolpix digital camera. On the final day the animals were sacrificed and whole thickness biopsies were taken from the wound skin interface for histological analysis.

7.4 Calculation of wound area

Digital images were analysed using the programme Image-Pro® Plus, Version 4.0 for Windows™ (Media Cybernetics, 8484 Georgia Avenue, Silver Spring, USA). The 1cm scale was also traced on each photograph, so that the wound area on each biopsy could be calculated in cm^2. This technique was validated by a comparison of two independent observers. The plot of observer 1 versus observer 2 gave a straight line with R = 0.995 (not shown).

7.5 Results

7.5.1. Qualitative results

The animals tolerated the procedure well with no wound infection. There was some loss of the split skin autografts in 2 wounds, about 20% in each. There

was complete loss of the Integra® in one wound and 50% loss in one other. The take rate of the ultra thin split thickness skin graft was 100% in three of the Integra® grafted wounds. However, two of these wounds had been those in which the Integra® had taken poorly or not at all. The take rate of the ultra thin split graft in the other three Integra® grafted wounds was 50%, 20% and 0%. It would be preferable to exclude any wounds which had any graft loss, but due to the small numbers involved in this pilot study the results were analysed in their pre-allocated groups despite this loss of some grafts. The open wounds contracted rapidly (**Figure 7.1**), and were almost completely closed by day 35. The wounds grafted with split skin grafts contracted more slowly (**Figure 7.2**), and the wounds grafted with Integra® and a thin split skin graft contracted at a variable rate depending on the take of both the Integra® and the subsequent graft (**Figure 7.3**).

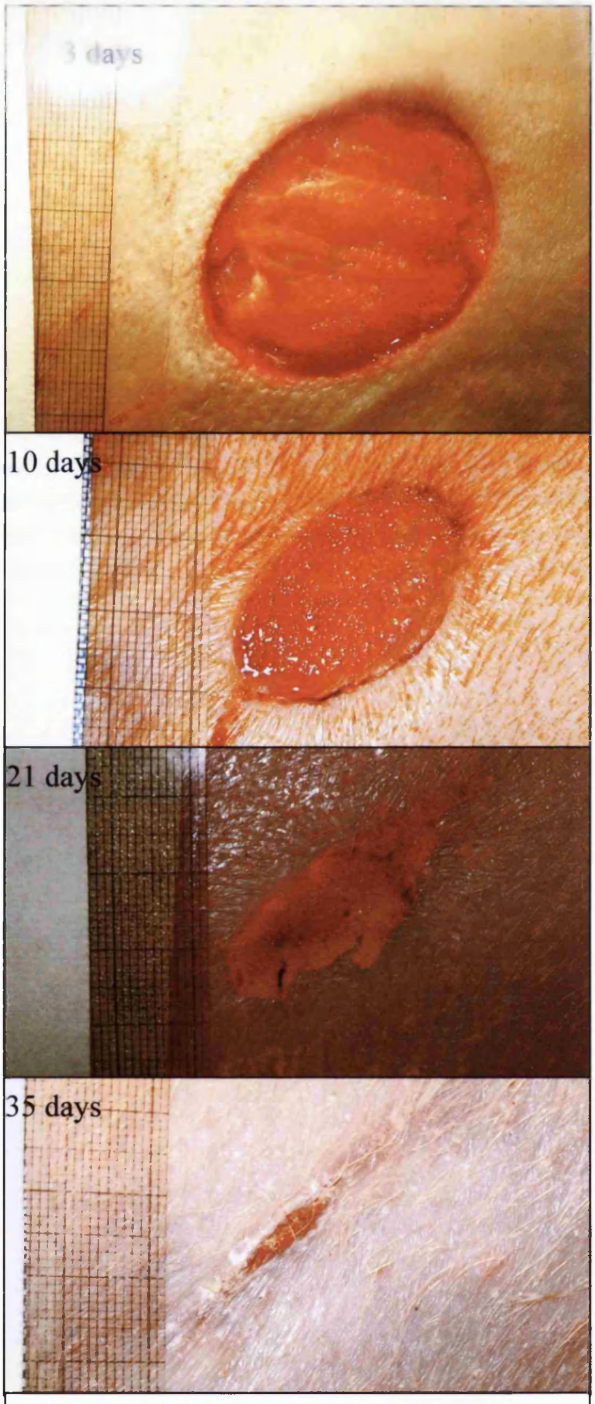


Figure 7.1 – The contraction of a full thickness skin wound
The wound was dressed with jelonet and gauze, and photographed at regular intervals. Each photograph has been put at a similar scale, the graph paper alongside the wound has 1cm bold markings. The wound has progressively reduced in size, until it is completely closed at day 35.

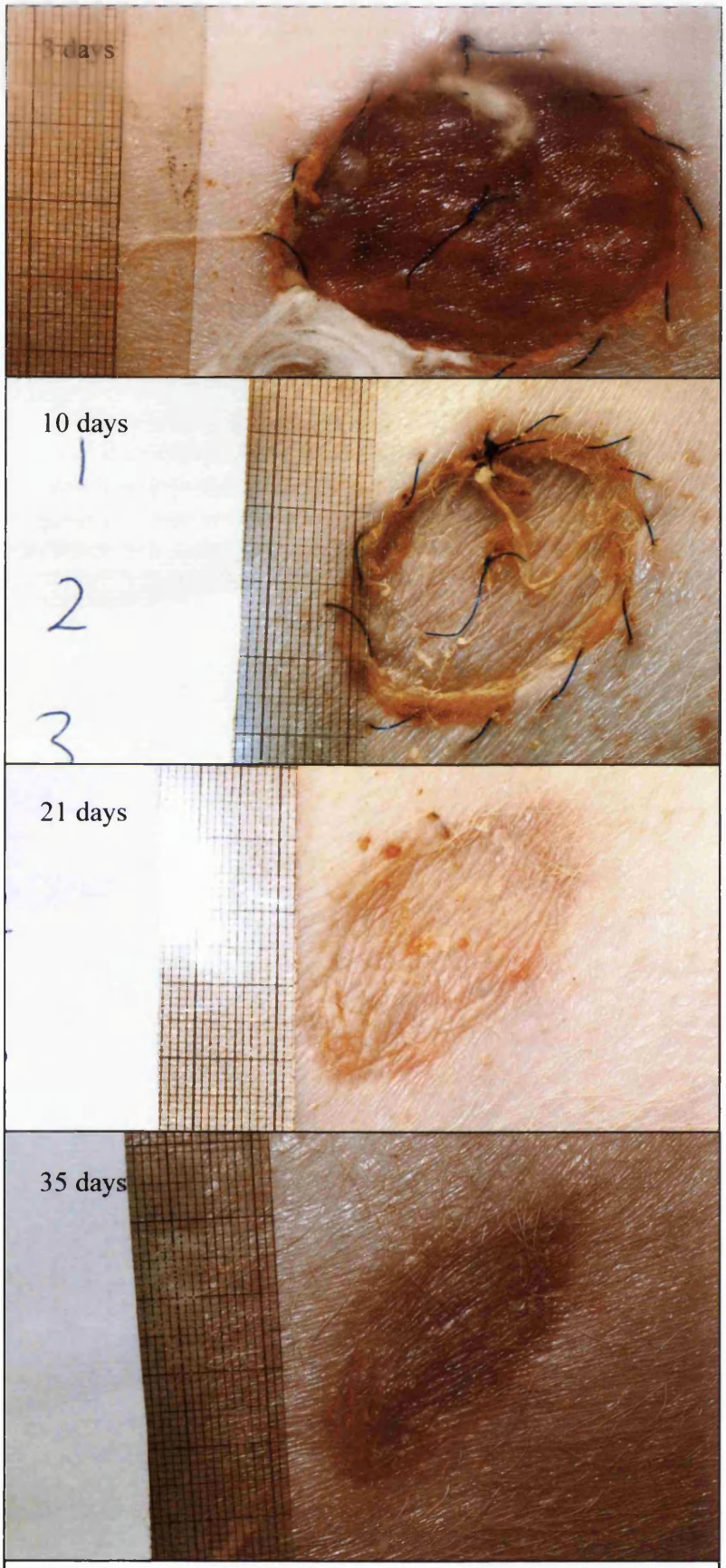


Figure 7.2 -The contraction of a full thickness skin wounds treated with a split skin graft.
The wound was photographed at fixed time intervals. There was 100 % graft take for this wound and contraction was small compared to the wounds which did not receive a graft (see figure 7.1)

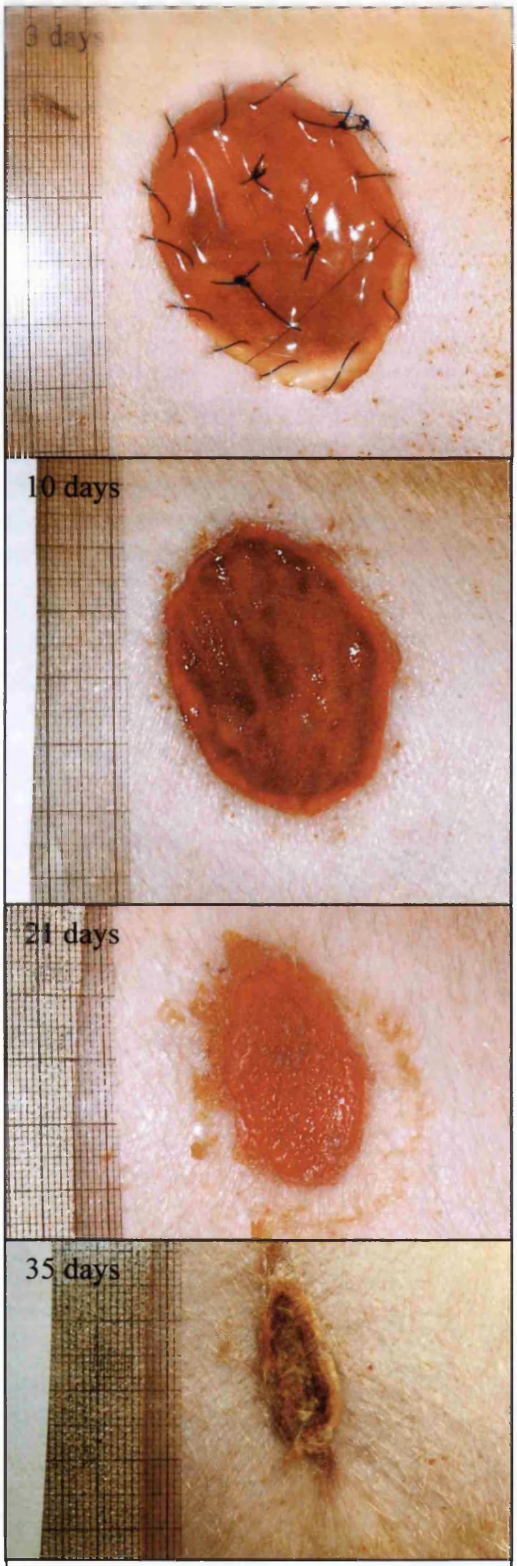


Figure 7.3 -The contraction of a full thickness skin wounds treated with Integra® with a split skin graft on day 10
The wound was photographed at fixed time intervals. There was 100 % take for the Integra® on this wound and contraction was initially small. However, the split graft had a poor take and the wound subsequently exhibited significant contraction.

7.5.2. Quantitative results

The graph of wound size against time is shown in **Figure 7.4**. In the first week the open wounds and the wounds grafted with split skin grafts contracted at the same rate. There was no statistically significant difference between the two groups (Mann-Whitney Rank Sum Test P = 0.699). The wounds grafted with Integra® contracted more slowly. There was a statistically significant difference between the open wound and the Integra® groups (t-test P = 0.037), and also between the skin graft and the Integra® groups (t-test P = 0.014).

At 10 days the open wounds had contracted the most, with the wounds grafted with split skin grafts contracting slightly less. The difference in the wound sizes was significant (P = 0.022, t-test). The wounds grafted with Integra® contracted even less than the wounds grafted with split skin grafts. The difference in wound sizes of the two groups was still significant (P = 0.002, Mann-Whitney Rank Sum Test).

The silicone was removed from the Integra® at ten days, and an ultra thin split skin graft was applied. The graph demonstrates an increase in the rate of wound contraction in this group, as demonstrated by a change in the slope of the graph. By 21 days there was no statistically significant difference between the wound sizes in the skin graft and the Integra® groups (t-test P=0.808). However, the wound sizes in both the skin graft and Integra® groups remained significantly different to the wound sizes in the open wound group (t-tests: Integra® group P = <0.001, skin graft group P = 0.005).

At the end of the study on day 35 there was still a significant difference between the size of the wounds in the open and grafted groups (t-test P = 0.002). However, there was no statistically significant difference between the size of the open wounds and the wounds grafted with Integra® (Mann-Whitney Rank Sum Test P = 0.240).

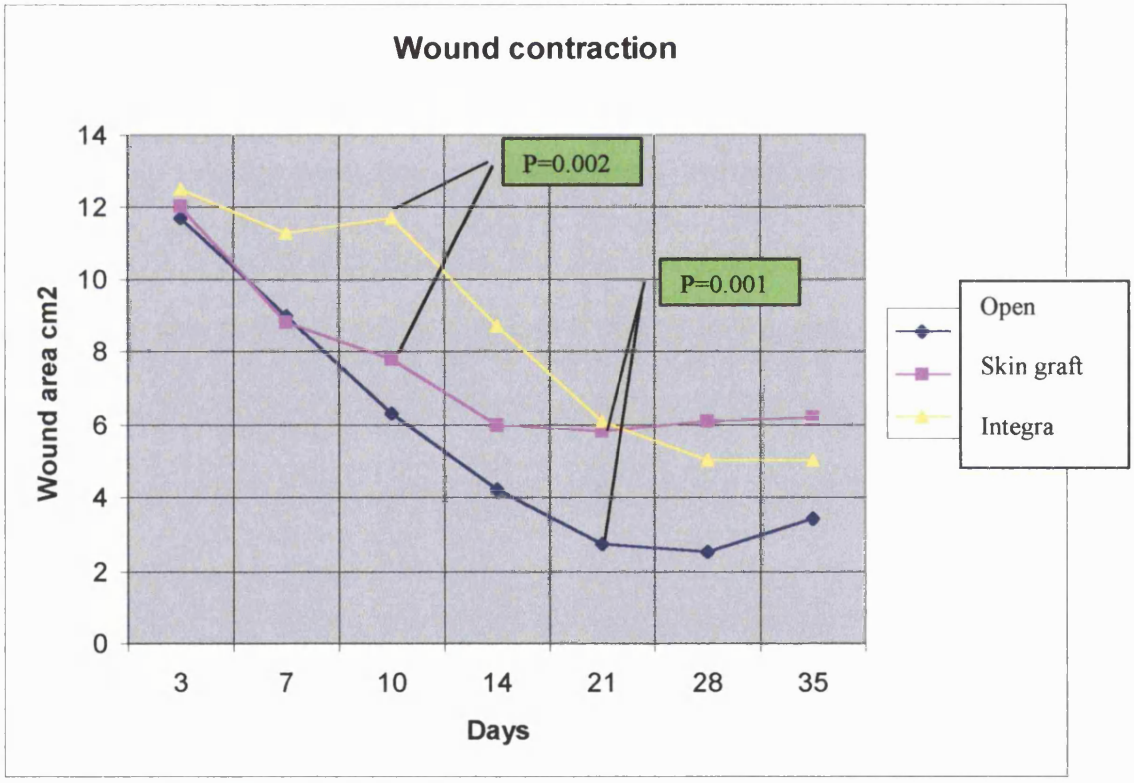


Figure 7.4 – Graph of mean wound size in cm^2 against time.
Six wounds were left open, six wounds were grafted with autologous split skin grafts and six wounds were grafted with Integra. The silicone was removed from the Integra at 10 days and an ultra-thin split thickness skin graft applied. The size of the wounds was measured using digital photography and image analysis at regular time points.

7.5.3. Histological results

The collagen deposition in the wounds left open was random (**Figure 7.5**). The wound surface was not completely re-epithelialised by 35 days.

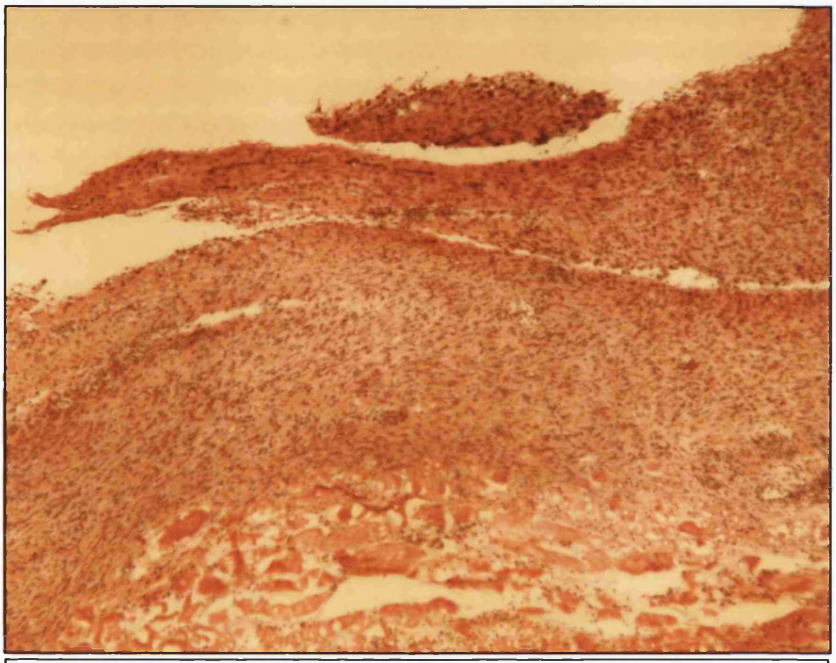


Figure 7.5 – H&E x 40 of un-grafted wound at day 35
There is still no evidence of epithelial formation and the wound bed is filled with disorganised collagen deposited in a random fashion

The wounds treated with split skin grafts had a normal epithelium, with two distinct types of collagen in the dermal layer (**Figure 7.6**). The superficial layer of collagen was orderly, similar to the collagen deposition of normal skin. Beneath this was a layer of disorganised collagen similar to that seen in the open wounds. The transition zone between the two types of collagen is seen in figure 7.6. Wounds treated with Integra® had a similar quality of epidermis to the split thickness skin grafts (the biopsy was from an area of good Integra®

take and good take of the ultra-thin split skin graft). There were once again two distinct areas of collagen deposition (**Figure 7.7**). The layer below the epidermis was orderly and similar to the collagen in normal skin. However, this layer was clearly thicker than the 150μm graft which had been applied, probably due to the concertina effect of the wound contraction. Beneath this layer of collagen was a layer of collagen which was not as orderly as normal skin collagen, but which was more orderly than the random deposition of collagen seen in the open wounds.

This correlates with the template action of the Integra® which is designed to produce orderly collagen deposition and create a neo-dermis. This can be clearly seen in **Figure 7.7**.

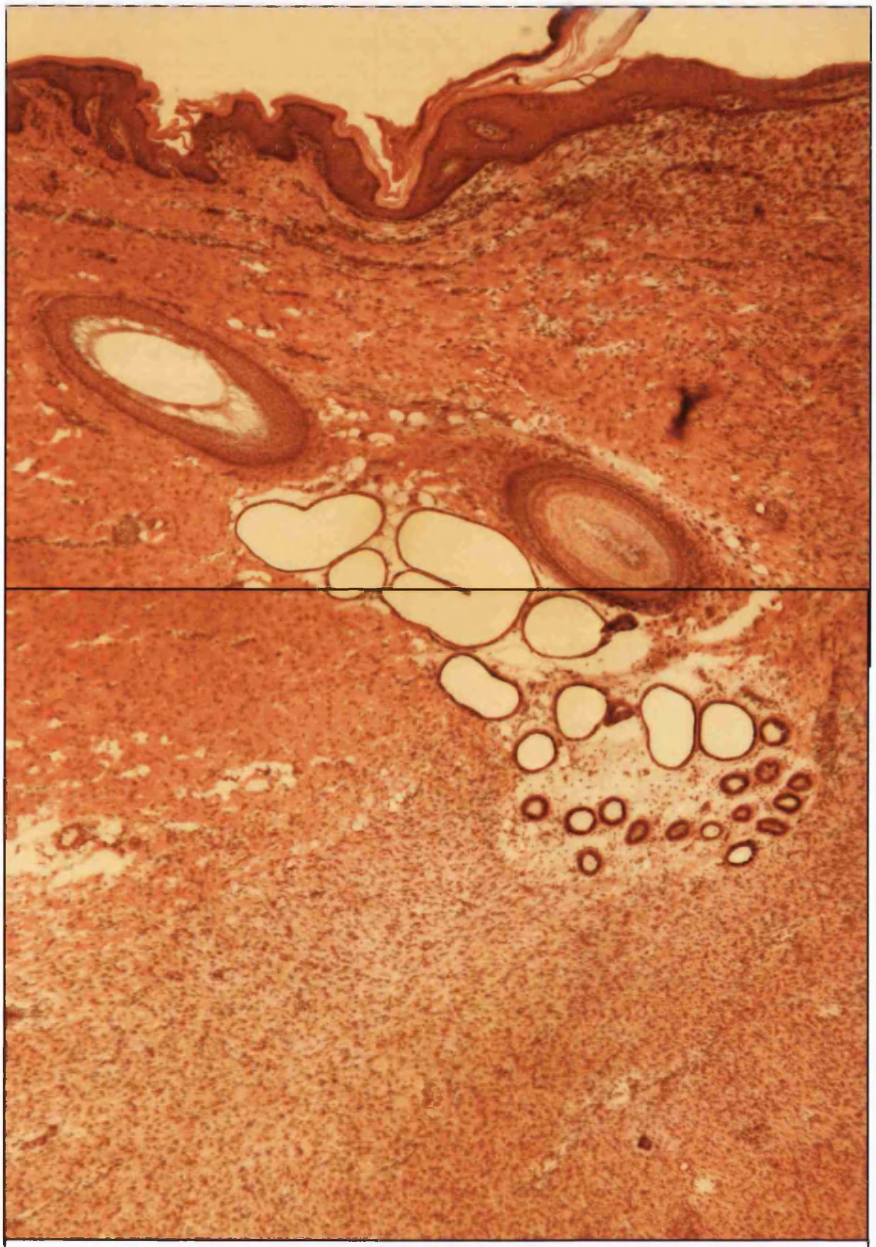


Figure 7.6 – H&E x 40 of wound grafted with a split thickness skin graft
The biopsy is taken at day 35 post grafting. The wound has two distinct layers of collagen beneath the epidermis. The first is an organised layer of reticular dermis which has been transplanted with the epithelium in the graft. The deeper layer of collagen is a more disorganised pattern of deposition similar to that seen in the open wound (see Figure 7.5). A hair follicle and associated sebaceous gland has been transplanted with the graft.

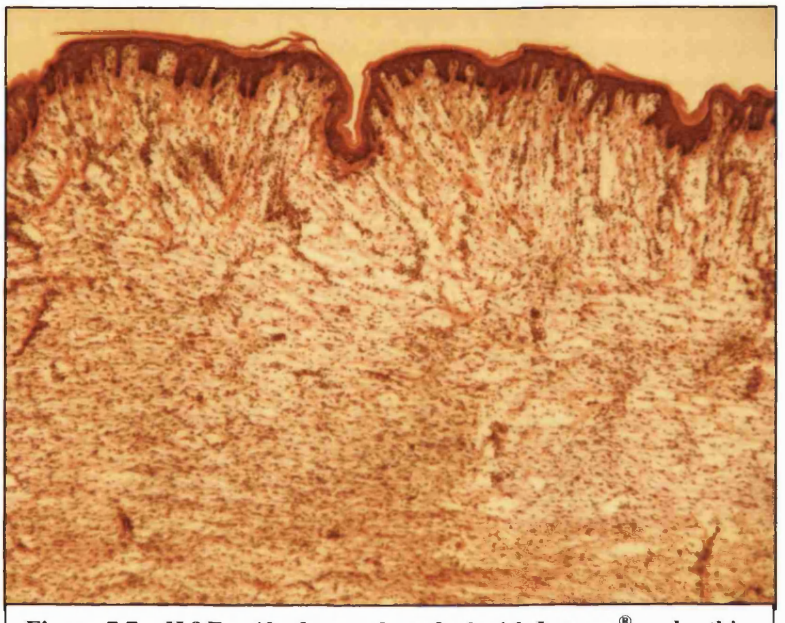


Figure 7.7 – H&E x 40 of wound grafted with Integra® and a thin split thickness skin graft
The biopsy is taken at day 35 post grafting. The wound has two distinct layers of collagen beneath the epidermis. The first is an organised layer of dermis which has been deposited around the Integra® matrix. The deeper layer of collagen is a more disorganised pattern of deposition similar to that seen in the open wound (see figure 7.5).

The Integra® take rate in this experiment was 75%. However, the rate of successful grafting of Integra® with subsequent successful grafting of an ultra-thin split skin graft was only 25%. The Integra® bio-integrates much more rapidly in pig wounds than in human wounds, which is why the silicone was removed at 10 days. However, it is possible that this was slightly too early, as incomplete bio-integration is the most likely explanation for the poor skin graft take in the absence of infection. The grafts have little chance of take if neo-vascularisation has not occurred in the Integra® at the wound surface.

Published take rates for Integra® are variable and are dependent on operator experience. A trial involving 106 patients from several centres found the median "take" was 85%, compared to take rates of 95% in the controls using split skin grafts. (Heimbach et al., 1988). Few authors comment on the subsequent take rates of the ultra-thin split skin grafts. The results of this study show clearly that the Integra® reduces the rate of contraction until the silicone outer layer is removed. Once the silicone is removed the wound contraction rates increased to a greater rate than that seen in wounds treated with only split skin graft at the corresponding time point. This is probably due to the poor take rate of the ultra-thin split skin grafts in this study. The wounds grafted with Integra® which had good split skin graft take appeared to contract less than wounds grafted with Integra® which had poor split skin graft take, although numbers were too small to divide these into statistical groups. The interesting point is that contraction rates appear to be controlled by "wound closure". The

silicone must physiologically close the wound, and factors within the dermal matrix must detect this physiological "closure" and prevent wound contraction. The silicone on Integra® is too thin to provide a mechanical splinting effect, and silicone is inert and incapable of producing contraction regulating cytokines. The fact that even wounds which had good take of the Integra® contracted significantly after failure of a split thickness skin graft, demonstrates that the contraction is not controlled by formation of a good quality neo-dermis.

Epidermis has a homeostatic barrier function. When intact it prevents foreign material entering a wound, but it also prevents loss of tissue constituents from a wound. It could be that increased wound contraction is the result of an absence of certain cytokine signals from an open wound. These may accumulate as soon as an epidermal barrier is formed, either in the form of a synthetic wound cover such as silcone or in the form of a squamous stratified epithelium. It is conceivable that such a control mechanism may effect the contraction rate and subsequent hypertrophic scar formation by influencing the rate of fibroblast apoptosis, with the accumulation of the cytokine signal triggering cell death. Further studies are required to examine these possibilities.

CHAPTER 8

INTEGRA® SEEDED WITH CULTURED AUTOLOGOUS KERATINOCYTES LABELLED WITH GREEN FLOURESCENT PROTEIN

8.1 Introduction

Green Fluorescent Protein has been used extensively as a marker for cell labelling studies both *in vitro* and *in vivo*. However, several studies have recently shown that the commercially available proteins used may be toxic to the recipient cells (Liu et al., 1999). Furthermore, there is also evidence emerging that the cells marked with these proteins are rejected by immuno-competent hosts (Doi et al., 2002). *LacZ* markers have already been used in Chapter 6. *LacZ* labelled cells can only be identified after histological preparation, and do not allow *in vivo* identification of labelled cells. The ability to insert a reporter gene, which can be identified *in vivo* confers obvious advantages in animal models. New variant green fluorescent protein reporter genes have recently been developed which claim to be less toxic to the recipient cells, and reduced rejection by immuno-competent hosts.

8.2 Study objectives

The aim of this study was to test a new variant of green fluorescent protein derived from the sea pansy *Renilla reniformis*. The gene for this protein has been humanised using codons preferred in highly expressed human genes. We aimed to test this marker in an established *in vivo* wound model for which substantial comparative data already exists (Chapter 3).

8.3.1. Preparation of virus producing cell line

The ViraPort™ retroviral reporter vector pFB-hrGFP (Stratagene), containing the humanised form of green fluorescent protein from the sea pansy *Renilla reniformis*, was used to transfect a RetroPack PT67 packaging cell line (Clontech) for high titer retroviral production. Co-transfection of 15µg of pFB-hrGFP and1.5µg pSV_2neo was carried out according to manufacturer's instructions using Lipofectamine Plus™ (Gibco) by Dawn Mann Blonde McIndoe Centre for Medical Research). Transfected producer cells were selected for the neomycin pSV_2neo resistant marker using 800µg/ml Geneticin (G418 Sulphate) (Gibco) 3 days after selection. Cells were continuously cultured under selective pressure in DMEM with Glutamax, Sodium Pyruvate and 4.5µg/ml glucose, and 10% foetal calf serum. Fluorescent colonies were isolated, expanded and screened for the production of the virus containing the hrGFP protein. PT67pFB-hrGFP clone A2 was re-selected using HAT supplements (Gibco) according to the Clontech protocol, to ensure retention of the viral genes.

8.3.2. Animals

A single animal was used for this study. Six full thickness wounds were created, three on either flank, down to skeletal muscle. PTFE chambers were inserted into the wounds (cf. 2.2.15) to prevent re-epithelialisation from the wound margin and wound contraction. Four wounds were grafted with Integra® seeded

with cultured autologous keratinocytes labelled with green fluorescent protein, and two wounds were grafted with Integra® seeded with unlabelled cultured autologous keratinocytes. One wound from each group was removed at week one, and the remaining wounds were harvested at week two.

8.3.3. Keratinocyte isolation and culture

Pig keratinocytes were isolated from split skin grafts (cf. 2.2.5). The autologous keratinocytes were cultured through four passages over a three week period, and then harvested from culture whilst still in a pre-confluent state. The autologous keratinocytes were counted with a haemocytometer, then centrifuged and re-suspended in keratinocyte growth medium in a concentration of 6.25 x 10^6 keratinocytes per ml for seeding into the Integra® in a volume of 2 ml.

8.3.4. Keratinocyte retroviral transduction

Second passage autologous keratinocytes were seeded into 75cm^2 flasks in a

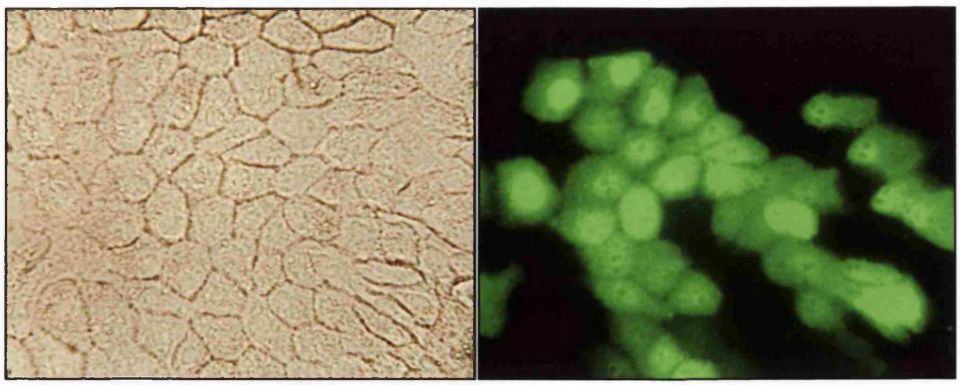


Figure 8.1 – Confluent pig keratinocytes after transduction with GFP
These pictures were taken of the same field at 100x magnification. The image on the left is under normal light, the image on the right is under ultra-violet illumination. The transduced keratinocytes expressing green fluorescent protein can be clearly seen.

density of $4x10^6$ per flask with 10ml of retrovirus containing keratinocyte culture medium. The keratinocyte medium was prepared in advance by incubation at 32°C for 72 hours with the PT67pFB-hrGFP A2 clone (without G418). The keratinocytes were incubated with the retroviral containing culture medium for 18 hours at 32°C. They were then rinsed and returned to fresh keratinocyte culture medium for culture through two further passages. The transduced keratinocytes can be seen in **Figure 8.1**.

8.3.5. Preparation of grafts

The grafts were prepared according to the protocol previously explained (cf. 2.2.16). The cultured autologous keratinocytes were stored in keratinocyte culture medium prior to their delivery into the Integra®. Keratinocytes were delivered into Integra® by dripping a suspension of cells onto the non-

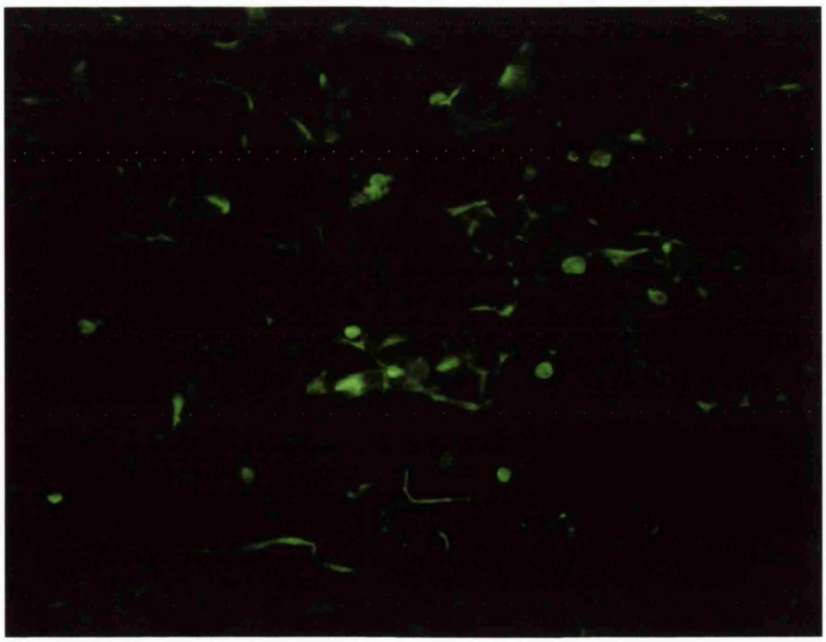


Figure 8.2 – Integra® seeded with keratinocytes labelled with GFP. The transduced keratinocytes were seeded onto the non-siliconised surface of the Integra®. This specimen was then fixed in formyl saline and 15μm paraffin sections prepared for viewing under ultra-violet microscopy. The transduced keratinocytes can be seen amongst the Integra® matrix which autofluoresces.

siliconised surface. Two grafts were prepared with non-labelled cells and four grafts were prepared with labelled cells. The average seeding density was $5x10^5$ keratinocytes per cm^2. Cell viability was 72% and 67% in the labelled and non-labelled groups respectively as assessed by a tryphan blue exclusion test. Additional grafts were prepared in the same way for histological analysis to ensure the cells were evenly distributed through the grafts (**Figure 8.2**)

8.3.6. Analysis of results

The whole wound excision biopsies were divided into quadrants and then prepared for histological section (cf. 2.2.18-21). However, as well as H&E slide preparation, some slides were prepared with antifade mountant only, as eosin fluoresces under UV light and interferes with image analysis. The slides were examined under UV light and compared with H&E examination under normal light.

8.4 Results

The animal tolerated the procedure well with no wound infections and complete take of the Integra® grafts. The unlabelled keratinocytes in the control wounds showed similar histological features to those seen in Chapter 3 (section 3.4.3). At one week the Integra® was uniformly infiltrated by proliferating keratinocytes. These were evenly distributed throughout the matrix in spherical accumulations consisting of 50-100 cells. By week 2 some of these accumulations had reached the surface of the Integra®, where they appeared to

open out and form an epidermis. No keratinocyte expressing green fluorescent protein were seen in any of the control wounds.

Flasks of transduced keratinocytes were examined for GFP activity using ultraviolet light microscopy to determine the transduction frequency of the GFP labelled cultures. The transduction frequency was 22% (**Figure 8.1**).

Keratinocytes expressing GFP could be seen on ultraviolet light microscopy in the four wound biopsies containing keratinocytes exposed to the GFP retrovirus. GFP expressing keratinocytes were distributed evenly throughout the Integra® matrix in the first week (**Figure 8.3 a and b**). The two-week biopsies show accumulations of keratinocytes in the Integra® matrix expressing GFP (**Figure 8.3c and d**). However, these keratinocytes appear to be differentiated compared to the keratinocytes in the control wounds at a similar time period (**Figure 8.3 c**).

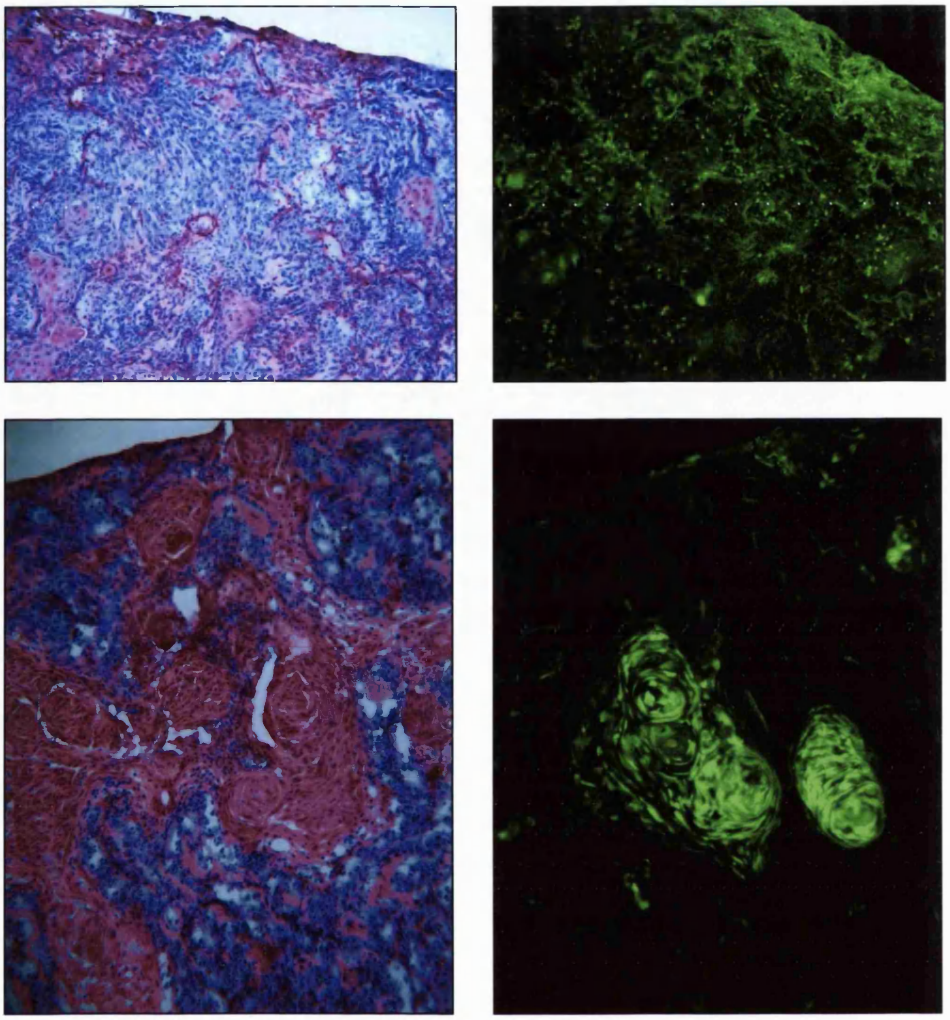


Figure 8.3 – Week 1 and 2 biopsies of GFP labelled keratinocytes seeded into Integra®

a) H&E of week 1 biopsy. The keratinocytes can be seen evenly distributed throughout the matrix (x 40).

b) UV of week 1 biopsy. The transduced keratinocytes can be seen throughout the matrix (no stain) (x 40).

c) H&E of week 2 biopsy. The keratinocytes have formed into collections or "cysts" within the matrix. They have taken on a differentiated morphology (x 100).

d) UV of week 2 biopsy. The transduced keratinocytes can be seen within the keratinocyte accumulations (no stain) (x 100).

No bright flourescence was seen with similar slides containing keratinocytes without the GFP reorter gene.

8.5 Discussion

We have prepared a retroviral producing cell line derived from the amphotropic PT67 packaging cell line by transfection with the ViraPort™ retroviral reporter vector pFB-hrGFP (Stratagene), containing the humanised form of green fluorescent protein from the sea pansy *Renilla reniformis*. We have used this retrovirus producing cell line to tranfect keratinocytes *in-vitro* and then transplanted the keratinocytes containing the reporter gene into an established wound healing model. The behaviour of the keratinocytes in this model has been established using monoclonal antibodies to cytokeratin 14 (cf. Chapter 3 section 3.4). We have found that although the keratinocytes can be successfully labelled with the GFP reporter gene, the *in-vivo* behaviour of these keratinocytes is different to keratinocytes not containing the GFP gene. The reason for this difference in behaviour is not clear. It may be that the GFP labelled keratinocytes are being recognised as "foreign" by the immunocompetant host and destroyed by cell mediated immune systems. However, the labelled keratinocytes may mature and differentiate prematurely as a consequence of the change in their DNA subsequent to retroviral transduction.

Another point of interest is the similarity on H&E sections in the cyst-like structures produced using GFP labelled keratinocytes seeded into Integra®, with the keratinocyte cysts formed when non-cultured keratinocytes were seeded into Integra® (cf. Chapter 4, figure 4.1). The "cysts" which formed within the matrix

when non-cultured keratinocytes were seeded into Integra® expressed keratin 10 and were probably due to the keratinocytes differentiating within the matrix, and losing their capacity to migrate through the matrix to the wound surface. The GFP gene may be having a similar effect on the cultured keratinocytes, causing them to differentiate within the matrix, forming similar "cyst-like" histological features.

It is clear that GFP labelling could be a useful tool for following the fate of keratinocytes both *in-vitro* and *in-vivo*, and further studies comparing the behaviour of GFP expressing cells in an immunocompromised model would be useful.

CHAPTER 9

GENERAL DISCUSSION AND CONCLUSIONS

9.1 General Discussion

The role of artificial skin substitutes in burn surgery and chronic wounds is constantly evolving. New products are regularly being produced and approved for clinical use. Studies on existing products are clarifying their efficacy and effectiveness in different clinical scenarios. Chapter 1 examines the components, structure, performance and comparative costs of the main commercial skin substitutes and reviews briefly technologies under development that have not yet become widely available. This material formed a major part of a recently published review (Jones, Currie & Martin 2002).

Integra® artificial skin is a commercially available skin substitute that is gaining widespread popularity for use in major full thickness wounds as well as for secondary burn reconstruction. However, there remain several difficulties associated with its application. The "take rate" can be poor, especially when associated with wound contamination. The technique still requires the use of a thin skin graft after bio-integration of the neodermis, with subsequent donor site morbidity. Techniques to replace grafts with cultured skin keratinocytes, which could be grown *in vitro* whilst Integra is undergoing biointegration, have had only limited success. The reasons for this are not yet understood. One possible approach has been to use actively dividing, sub-confluent keratinocytes rather than sheets of epidermis that may contain cells with poorer adhesive and replicative characteristics. There are two main approaches to employ sub-confluent cells. One is to spray suspensions of sub confluent cells to the wound

bed the second is to seed the underside of the Integra matrix with a cell suspension before the material is grafted.

In this research program the Large White Pig was used as an animal model to investigate aspects of both Integra® seeding and the sprayed application of cells. The model has the option to employ wounds isolated in PTFE chambers to prevent wound contraction and marginal re-epithelialisation. One strategy to improve the efficiency of sub-confluent cell suspensions is to use some kind of biomaterial delivery matrix. Fibrin sealants are an obvious choice since they are already in clinical use and fibrin is the natural provisional matrix in the early stages of wound healing. A review of the use of fibrin sealant in skin grafts and tissue engineered skin replacements was carried out. Fibrin sealants do have some properties such as improved haemostasis and antibacterial activities that would be attractive for use in burns and reconstructive surgery. This review was subsequently published (Currie, Sharpe & Martin 2001).

This research program examined the use of sub-confluent keratinocytes when seeded into Integra® artificial skin or as sprays onto the wound bed and looked for evidence that cultured cells were in fact of greater utility than non-cultured skin suspensions. In both cases the benefit of using fibrin sealant was examined. In addition, the importance of epidermal cover on the rate of contraction of Integra® artificial skin was investigated. Preliminary investigations on cell tracking of fibroblasts and keratinocytes with LacZnls and hrGFP gene markers were conducted in collaboration with other investigators. The research questions that informed the investigations in this program of work

were formulated as a series of hypotheses at the end of chapter 1. Individual studies that tested specific hypotheses form Chapters 3, 4, 5, 6 & 7. Detailed discussions are found at the end of each chapter but the outcome of each hypothesis is reviewed below.

When these wounds were grafted with Integra® seeded with cultured autologous keratinocytes, the keratinocytes were shown to migrate through the matrix and only differentiate once they reached the surface. The keratinocytes produced a 20.4% epithelial cover by three weeks. When the cultured autologous keratinocytes were suspended in the fibrin component of fibrin glue and then seeded into Integra®, a 42.1% epithelial cover was produced by three weeks (though this difference was not significant P= 0.097). The use of fibrin glue also improved haemostasis of the wound bed and made suture of the matrix technically easier.

When non-cultured autologous keratinocytes were used to seed the Integra® the keratinocytes failed to migrate through the matrix. Instead they formed keratinocyte cysts which expressed keratin 10, a marker of differentiation. Keratin 10 was not expressed when cultured keratinocytes were used, and it was postulated that the non-cultured keratinocytes contain a high percentage of paraclones compared to holoclones. The paraclones have a short replicative lifespan and differentiate before reaching the wound surface, thus losing their migratory capacity and forming cysts. Similar cysts were seen when cultured autologous keratinocytes were labelled with a "Green Fluorescent Protein"

reporter gene. The new DNA or some process within the transduction procedure may be causing early differentiation.

When cultured autologous keratinocytes were sprayed directly onto a freshly debrided wound isolated by a PTFE chamber, without Integra®, an epithelium was formed over 25.8% of the wound surface. The addition of fibrin glue to the sprayed suspension did not improve the keratinocyte "take rate" (P=0.802).

Cultured autologous and allogenic fibroblasts labelled with a *lacZ* reporter gene and then seeded into Integra® survived *in vivo* for two weeks, but could not be seen after this time. More autologous fibroblasts were seen at two weeks than allogenic fibroblasts. However, the fibroblasts seeded into the matrix were insignificant compared to the number of fibroblasts derived from local mesenchymal cells which migrated into the matrix within the first week. The labelled fibroblasts survived for at least six weeks *in vitro.* The fibroblasts may be recognised as foreign due to the altered DNA content, accounting for the reduced survival *in vivo*. Alternatively they may be present in numbers too low for detection.

9.2 Analysis of hypotheses

1. **<u>Cultured</u> autologous keratinocytes when seeded into Integra® artificial skin will produce an epidermis, with migration of keratinocytes through the matrix towards the wound surface.**

This hypothesis is supported (Chapter 3).

The graph in **Figure 3.21** demonstrates a gradual reduction in the number of keratinocytes within the matrix, and the graph in figure 3.20 demonstrates a gradual increase in the number of keratinocytes at the wound surface. This supports the hypothesis that the keratinocytes migrate through the matrix to the wound surface.

2. **<u>Non-cultured</u> autologous keratinocytes when seeded into Integra® artificial skin will produce an epidermis, with migration of keratinocytes through the matrix towards the wound surface.**

This hypothesis is rejected (Chapter 4).

Figure 4.2a and **b** show that this same process of migration does not occur in a similar way when non-cultured keratinocytes are used to seed the matrix. Cysts are formed within the matrix, as shown in **Figure 4.1**.

3. **Fibrin glue will improve both the efficiency and efficacy of the delivery of subconfluent cultured autologous keratinocytes into the tissue engineered dermal replacement Integra®.**

This hypothesis is only partially supported (Chapter 3).

When fibrin glue was used to seed the Integra® with cultured autologous keratinocytes the matrix was easier to suture due to the instant adhesion to the wound bed. There was also a subjective improvement in the wound bed haemostasis. The fibrin glue did not prevent keratinocyte migration through the

matrix and there was a trend towards an increase in epithelial cover at three weeks associated with the use of fibrin glue (**Figure 3.2**).

4. **Fibrin glue will improve the take rate of Integra® artificial skin when used as a keratinocyte delivery system, due to its adhesive, haemostatic and anti-bacterial effects already demonstrated when used with skin grafts.**

This hypothesis is neither supported nor rejected (Chapter 3).

The take rate for Integra® with or without fibrin glue was very high in this experiment, 86% overall. Therefore it was difficult to show any improvement with the use of fibrin glue. However, the fibrin glue did not interfere with the Integra® take (cf. 3.4.4.2).

5. **Fibrin glue will improve the "take" of sub-confluent cultured autologous keratinocytes delivered to a wound bed in a spray-on-suspension.**

This hypothesis is rejected (Chapter 5).

Figure 5.5 and **5.4** show no significant difference in the keratinocyte take rate when sprayed onto a wound bed in fibrin glue or culture medium (P=0.802 and 0.878).

6. **Labelled <u>autologous</u> fibroblasts seeded into Integra® artificial skin will contribute to the fibroblast population in the regenerating dermis.**

This hypothesis is supported up to the two weeks, after this time it is neither supported nor rejected (Chapter 6).

Labelled autologous fibroblasts were detected in the wounds at two weeks (p136) but were not detected at three or four weeks. This may be due to the cells being present in numbers too small for detection compared to the massive influx of native fibroblasts from the surrounding non-wounded tissue, or due to cellular destruction as a result of the foreign DNA content from retroviral transduction.

7. **Labelled allogenic fibroblasts seeded into Integra® artificial skin will contribute to the fibroblast population in the regenerating dermis.**

This hypothesis is supported up to the two weeks, after which time it is neither supported nor rejected (chapter 6).

Labelled allogenic fibroblasts were detected in the wounds at two weeks (**Figure 6.3**) but were not detected at three or four weeks. This may be due to the cells being present in numbers too small for detection compared to the massive influx of native fibroblasts from the surrounding non-wounded tissue, or due to cellular destruction of the allogenic cells by the hosts immune system.

8. **A wound grafted with Integra® artificial skin and covered with an ultra-thin split skin graft will contract less than an open wound or a wound grafted with a split skin graft alone.**

This hypothesis is rejected (Chapter 7).

Figure 7.4 demonstrates that the Integra® contraction rate was less than that seen with open wounds and wounds closed with a split skin graft for a period of 10 days. However, when the silicone outer layer was removed the wounds grafted with Integra® contracted at a greater rate than the open wounds or the wounds closed with split skin grafts.

9.3 Further potential studies

1. **Seeding of Integra® with cultured autologous keratinocytes** – This technique has been shown to be successful within the pig wound model. Its application now needs to be extended to humans. Patients with major full thickness burns (over 50% of total body surface area) have no suitable alternative for wound closure, and at present the burn wounds are temporarily closed with cadaveric allograft until the split skin graft donor sites can be re-harvested. This technique could provide definitive wound closure at three weeks.

2. **Seeding of Integra® with cultured autologous fibroblasts** – There was a subjective improvement in the take of the split skin graft used to cover the Integra® when it had been seeded with cultured autologous fibroblasts. This may warrant further investigation. It would also be useful to repeat the seeding of labelled fibroblasts using a higher seeding density, which may improve detection after two weeks.

3. **Spray application of cultured autologous keratinocytes** – The sprayed keratinocytes have now been shown to contribute to wound closure in a pig

wound model when sprayed onto muscle fascia, forming an epithelium with a basement membrane. However, despite widespread clinical use, sprayed autologous keratinocytes have not been tested in any credible clinical trial to promote rapid wound closure, reduce morbidity or mortality, or improve cosmetic outcome. These clinical studies need to be carried out to justify the costs and the potential risk of xenogenic infection from foetal calf serum or mouse 3T3 cells.

4. **Contraction of artificial skin substitutes** – This thesis investigated the contraction rates of Integra® in comparison to open wounds and split skin grafts. This was only a pilot study due to time constraints and there is scope for repetition of this experiment to increase the data, especially as "take" rates were poor. The role of silicone within the Integra® is of interest. When this is removed the contraction rate increased considerably, and the mechanism behind this would merit further investigation.

9.4 Presentations and Papers arising from this Thesis

Publications

1. Jones I. **Currie L.J.** Martin R. **A guide to biological skin substitutes**. *Br.J.Plast.Surg.* 2002;55: 185-193.

2. **Currie, L. J.,** Sharpe, J. R., and Martin, R. **The use of fibrin glue in skin grafts and tissue-engineered skin replacements**: A review. *Plast Reconstr.Surg.* 2001; 108: 1713-1727.

Presentations at scientific meetings

1. **The Wound Healing Society 12th Annual Symposium and Joint Conference with the European Tissue Repair Society - Baltimore, USA. May 2002**. Integra seeded with non-cultured keratinocytes: The selective advantage of keratinocyte culture. Currie L. Martin R.

2. **British Burns Association – Birmingham. May 2002.** Keratinocyte cell sprays with and without fibrin glue. **Currie L.** Martin R.

3. **European Tissue Engineering Society Annual Meeting- Freiburg, Germany. Nov 2001.** Cultured autologous keratinocytes seeded into Integra® and the benefits of fibrin glue. **Currie L.** Martin R.

4. **British Association of Plastic Surgeons Winter Meeting- London. Nov 2001.** Integra® seeded with fibrin glue cultured autologous keratinocyte suspensions. **Currie L.** Martin R.

5 European Tissue Repair Society 11th Annual Conference- Cardiff Sept 2001. Artificial skin substitutes seeded with fibrin glue cultured keratinocyte suspensions. **Currie L.** Martin R.

Posters at scientific meetings

1. **The Wound Healing Society 12th Annual Symposium and Joint Conference with the European Tissue Repair Society– Baltimore, USA. May 2002.** A comparison of cultured autologous keratinocyte cell sprays with and without fibrin glue. **Currie L,** Martin R.

2. European Tissue Engineering Society Annual Meeting- Freiburg, Germany. Nov 2001. The *in vivo* fate of cultured autologous and allogenic fibroblasts. Mann D, Currie L, Martin R.

Papers in review

1. **Currie L.J.** Martin R. **The classification of tissue engineered skin replacements**.

2. **Currie L. J.** Martin R. **The use of fibrin glue to seed Integra® artificial skin with cultured autologous keratinocytes**.

3. **Currie L.** Martin R. **Keratinocyte cell sprays with and without fibrin glue**.

4. **Currie L.** Martin R. **Integra seeded with non-cultured keratinocytes: The selective advantage of keratinocyte culture.**

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