THE UNIVERSITY OF QUEENSLAND

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Individual Inquiry Topic A

Tissue Engineering

Skin:

Regeneration After

Full-Thickness

Wounds

FINAL REPORT

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Abstract

This report considers the repair of full-thickness wounds. The anatomy and aetiology of the wound is introduced, and current treatment procedures are considered. A discussion of the advantages and disadvantages of recently developed potential treatment procedures precedes a proposed skin regeneration approach. The strategy believed to be best suited to the task is proposed. For this strategy an in vivo approach has been adopted, involving the implantation of a synthesised bovine collagen extra-cellular matrix seeded with cultured allogenic fibroblasts. Gelatin microspheres are to be utilised for the controlled delivery of basic fibroblast growth factor at the wound site.

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1 PROBLEM STATEMENT

The skin is the body's largest organ, and one of the least appreciated. It provides the body with its first line of defence against both infection and dehydration. It also has a pivotal role in temperature control, increasing the rate of heat loss by routing blood close to the surface and exuding sweat, or decreasing heat loss with the aid of hair follicles as appropriate. Unfortunately its position at the exterior of the body means that it is vulnerable, and is frequently damaged. The ability of the skin to repair itself following minor injury is remarkable, but when the injury is severe, medical intervention is required, both to speed the recovery of the skin itself and to protect the body from infection and fluid loss in the meantime. The burn is one of the most common types of skin damage to require medical attention.

There exist four major types of burn: thermal, chemical, electrical and radiation (sunburn is a type of radiation burn). The most common burn type requiring medical treatment is the thermal burn. Burns are classified according to severity, as follows:

1. First-degree burns only affect the epidermis. These are painful, but heal rapidly, and generally do not require medical attention.
2. Second-degree burns involve damage to both the epidermis and the dermis.
3. Third-degree burns are burns for which the skin has been damaged or destroyed to its full depth, and may also involve damage to underlying tissues.

1.1 Socio-Economic Impact

Approximately 2,000,000 burns per year in the United States require medical attention[1]. Of these, about 70,000 require hospitalisation, and 20,000 entail referral to a specialized burns centre. Burns enable viruses and bacteria to breach the body's defences at a time when it is most vulnerable, and about 10,000 patients die each year of infections subsequent to sustaining serious burns. This vulnerability to infection often dictates long-term admission to an intensive care unit, at a very high cost.

2 ANATOMY AND AETIOLOGY

2.1 Anatomy of Skin

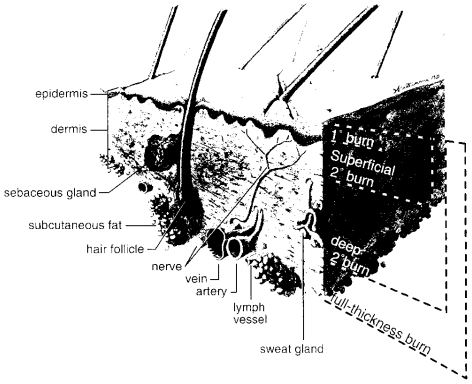

Fig. 2.1 Diagram of the skin including description of various depths of burn injury[2]

The skin is a stratified epithelial tissue, which is derived from the ectoderm layer of the embryo[2]. It consists of two layers, which are the epidermis and the dermis. The epidermis is the upper layer of the skin, which provides the body with its first line of defence. The basal layer of keratinocytes is located adjacent to the basement membrane that comprises the dermo-epidermal junction. Keratinocytes are rapidly dividing stem cells, responsible for the generation of epidermal cells that differentiate and mature as they move outwards. Of necessity, cell turnover is rapid, as cells must be readily replaced when they are lost.

The dermis is the living layer that acts as a substrate and a support network for the epidermis. It is differentiated into various components such as sebaceous glands, sweat glands, nerves and hair follicles. While this differentiation is difficult to replicate during repair after a severe injury, the many cell types are not essential to the correct functioning of skin.

The essential dermal cell type is the fibroblast, which is responsible for the production and maintenance of the structural elements of skin[2]. These elements, which include collagen and elastin, combine with non-fibrous substances such as glycosaminoglycans (GAGs) to form the extra-cellular matrix (ECM). The ECM also supports the basement membrane, ensuring the integrity of the dermo-epidermal junction[3]. Organized tissue renewal depends on the ECM. Normally, turnover of collagen is low, but occurs at a higher rate during damage repair.

The vascular network, which is difficult to replace, is quite critical to skin regeneration. Without an adequate blood supply, repair is inhibited, and if revascularization cannot be achieved, scar tissue is the only tissue type that can be supported.

Scar tissue is preferable to no healing but has a number of faults which justify minimising its formation where possible:

- Fully cross-linked, it only has 70% of the tensile strength of the tissue it replaces
- It is not fully functional, and is undifferentiated
- Scar tissue is aesthetically disfiguring

2.2 Aetiology of the burn wound

As mentioned earlier, there are several possible causes for burn wounds, but regardless of the cause, the treatment is generally similar[2]. All burns result from the destruction of tissue, but only the most common burn type will be discussed here: the thermal burn. Heat can be transferred to the skin by three methods: conduction, convection and radiation. Any of these three methods can result in a burn, but direct contact (conduction) damages the skin most rapidly (fig. 2.2).

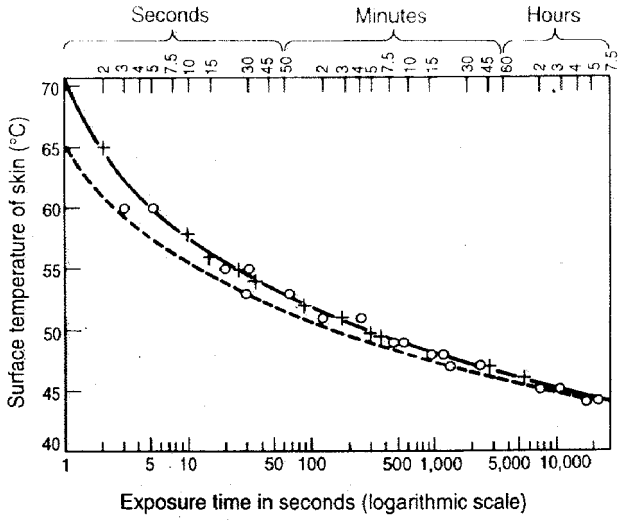

Fig. 2.2 Time-surface temperature thresholds at which cutaneous burning occurs[2]*. The dotted line indicates the threshold of irreversible epidermal damage, while the solid line indicates the threshold of epidermal necrosis.*

Two factors determine the severity of a burn: the temperature to which the skin is exposed, and the time for which the temperature is maintained[2]. It is possible for burns to occur at surprisingly low temperatures: experiments have demonstrated that irreversible damage can occur at temperatures as low as 44°C. It is above this temperature that the damage accumulation rate exceeds the natural repair mechanisms of the cell. As can be expected, the time for which a cell must be exposed to this temperature before necrosis occurs is great, but this decreases steeply as the temperature is raised.

Damage severity also increases with temperature: for a sufficiently great temperature, denaturation is so complete that all levels of protein structure, including the ECM, are destroyed. This is known as coagulation. The flow of blood to the site is also impaired by destruction of blood vessels, small blood clots and vascular constriction, which compounds the reduction in oxygen supply to the wound site.

After the wound is sustained, exudate is supplied to the site by the body[3]. This contains all constituents necessary for complete regeneration of skin, including fibroblasts, growth factors and nutrients. However, in the absence of the guidance provided by the ECM, the repair mechanism lacks direction, and the best result that can be expected is scar or fibrotic tissue.

3 EXISTING TREATMENT

3.1 Natural repair

Natural wound healing typically consists of three phases: Inflammation and Debridement, Repair and Maturation. (*The information in section 3.1 was taken from* Purves *et al*, *Life: The Science of Biology*[4] *unless otherwise referenced*)

1. *Inflammation and Debridement*

   Immediately a wound is sustained, the body enters damage control. Platelets in the blood are activated by contact with collagen outside the blood vessels, which causes them to adhere to each other (the blood coagulates). They also release chemicals, which encourage both vascular constriction to stem blood flow and platelet aggregation to enlarge clot and plug the wound. White blood cells are mobilised to fight sources of infection as they enter the wound, so capillaries surrounding the wound become engorged. This engorgement has the effect of increasing the permeability of the capillary walls. Lytic enzymes remove dead tissue to speed the healing process. The length of the inflammation and debridement phase varies, and is prolonged by infection, lack of blood supply to the wound site and the obstruction by necrotic tissue.

2. *Repair*

   The repair phase normally begins within twelve hours of the wound occurrence. This involves granulation, fibrification and epithelialisation. Granulation involves the formation of a scab, which acts as a scaffold for new cells to attach to. Depending upon the severity of the wound, fibrification may be required. Fibroblasts, which produce fibrous tissue, enter the wound site during fibrification. Fibrous tissue acts as a barrier against infection, and is required for wound contraction, during which intact tissue around the wound contracts to bring the sides of the wound together. Revascularization occurs to some extent during fibrification. Once a scaffold is in place, epithelialisation can take place: the migration and multiplication of cells to form new tissue. The presence of infection, too much granulation, hypothermia and insufficient blood supply are all factors that protract the repair phase.

3. *Maturation*

   Depending upon the severity of the wound, maturation takes between 14 days and 12 months to complete. The fibroblast concentration at the wound site is reduced, and fibroclasps enter the site. Fibroclasps produce lytic enzymes that remove the irregularly arranged collagen laid down during the initial phases. The wound site is then strengthened through regeneration of the extra-cellular matrix. This is achieved in part by the laying down and cross-linking of correctly oriented collagen fibres by fibroblasts.

Scarring occurs when the damage is so extensive that an adequate support system cannot be regenerated. That is, the blood vessel network is not replaced. Scars typically exhibit an

inverted triangular cross-section, as epidermal tissue encroaches from each side above the necrotic dermis, leaving a thin line of scar tissue visible at the surface.

3.2 Standard Treatment

First aid for all burns consists initially of cooling the wound (typically with cold water), as damage accumulation will continue to occur until the wound is cooled to within a tolerable temperature range[2]. Rapid cooling minimises the damage that occurs, and plays a large part in determining the extent and depth of the burn. Dressing the wound is also important from the point of view of preventing both infection and fluid loss.

To date, the standard treatment for severe burns has centred on the grafting of skin harvested from an undamaged part of the patient's own body (known as an autograft)[5]. This method works reasonably well for the replacement of the epidermis, provided the damage is not too extensive and sufficient undamaged skin is available. Usually, the skin is taken from the same location on several occasions, and is allowed to regrow between harvesting. This procedure is slow, and results in a 'chequerboard' effect both at the donor site and the burn site. It has been found that burn healing can be accelerated by a number of factors, including:

- The application of pressure to the burn site, which encourages the rapid reinforcement and cross-linking of the new tissue. This serves two purposes: it increases the strength of the skin, and it improves aesthetic appearance by providing an environment that promotes correct healing and minimisation of scar tissue.
- Application of topical antibiotics to the wound site to prevent infection of the wound and allow the body to concentrate on wound healing rather than fighting disease.

Alternatively, it is possible to source skin either from other people or cadavers (an allograft), or from an animal such as a cow or a pig (a xenograft)[5]. The body's immune response means that these grafts are almost inevitably rejected, so such grafts serve only to buy time while a permanent solution is sought. It is generally not possible to administer immunosuppressive drugs to prevent rejection of foreign skin grafts, as is normal procedure during the transplantation of any other organ, as infections are common, and the immune system is required to be fully functional. This means that alternatives such as autografts, or the transplantation of an acellular collagen matrix (free from cells that would cause it to be rejected) must be implemented.

Living tissue has an extremely limited shelf life outside the body, and preservation techniques such as freezing must be used if the tissue is to remain viable. The growth of epidermal tissue on the body as opposed to externally has the potential to defeat this problem, as well as to greatly decrease the costs associated with the growth factors and other nutrients required for growth in a laboratory.

3.3 Commercial Products

3.3.1 *Integra™*

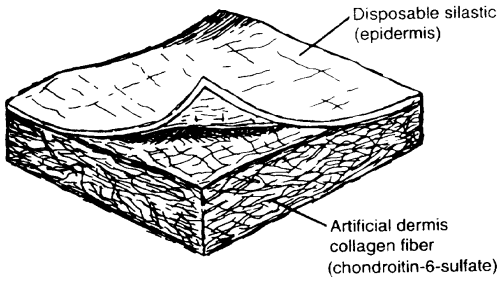

Fig. 3.2.1 Schematic diagram of Integra™ [2]

Integra™ is an artificial skin, which consists of two layers[6]. The lower layer is made up of an ECM of synthetically cross-linked bovine collagen, forming a scaffold to which the dermal cells may attach. The upper layer consists of a flexible silicon sheet to protect the site. Once the dermis is established, the upper layer can be removed, and depending upon the size of the wound, epidermal cells (either an autograft or an allograft) may be applied. Integra has a take rate of 80%, compared with 95% for an autograft. One major advantage of Integra over autografts is the reduced tissue thickness that must be harvested from donor sites, allowing more rapid healing both at the burn site and at the donor site. However, it should be noted that Integra does not provide any assistance to epidermal regeneration.

3.3.2 LifeCell™

LifeCell[7] have patented a method of removing cells from human dermal tissue while maintaining the collagen matrix, under the trade name AlloDerm®. This is achieved through chemical processing. The patent also extends to include a method of freeze-drying this ECM without damaging the structure, for the purposes of preservation. This gives AlloDerm a shelf life of approximately one year, but the product sells for about $10,000 per square foot[8]. AlloDerm functions in a similar fashion to Integra. Arguably, healing is assisted by the fact that the collagen is arranged in the form of a true human ECM.

LifeCell has a major disadvantage – its raw material is cadaver skin, the supply of which is limited. There are also safety issues associated with the harvesting of the skin, which must be performed soon after death to prevent sepsis from taking hold.

3.3.3 Epicel™

Epicel™ [9] differs from the previous two treatment methods in that epicel is only suitable for replacing the epidermis. Epicel involves the culturing of a small sample of the patient's skin *in vitro* until it is large enough to be meshed and grafted onto the patient. Meshing the skin prior to application greatly increases the area that can be covered with a given graft. The nutrients and growth factors required for epidermal regeneration are supplied by a substrate of mouse fibroblasts that have been lethally irradiated to ensure that they do not divide and multiply themselves. The utility of Epicel is limited by its inability to assist dermal regeneration, which is frequently a more pressing concern than epidermal replacement. As a

living tissue, the shelf life of Epicel is just 2 hours, and the cost is $15,000 per square foot[8]. However, there exists a very useful potential for the epidermis to be cultured in parallel with dermal regrowth. Total healing time, along with the risk of infection, can thus be greatly reduced.

3.3.4 Dermagraft™

One of the major disadvantages of many grafts is their lack of mechanical strength; hence the approach taken by the developers of Dermagraft[10]. Essentially, this is a polyglactin-910 mesh, seeded with autologous fibroblasts. Polyglactin-910 is the polymer from which biodegradable sutures are made. The benefits of increased mechanical strength include

- Ease of handling avoiding the requirement for a backing material
- Ability to hold sutures and staples

An interesting characteristic of dermagraft is the lack of an ECM. The fibroblasts seeded in the mesh secrete the proteins, glycoproteins and collagen necessary for the formation of the ECM *in vivo*. This avoids the susceptibility of the collagen matrix to both collagenases and microorganisms in the wound bed.

The fibroblasts are essential for healing, however. It was demonstrated that when the mesh was grafted to the wound when not seeded with fibroblasts, ingrowth of fibrovascular tissue from the wound bed did not occur, and the mesh rapidly separated from the wound.

A disadvantage of this approach is the need for autologous fibroblasts, which means that this treatment may not be administered until the requisite cells have been separated and cultured. However, the low immunogenicity of fibroblasts is considered in section 4.5 of this report.

4 POTENTIAL TREATMENT PROCEDURES

There exist a number of potentially successful treatment procedures, all of which utilise an extra-cellular matrix to provide a template for repair. Variations on the repair procedure revolve around modification of this matrix to promote repair. The structural and mechanical properties of the matrix can be altered during the matrix synthesis phase, controlling factors such as the degree of cross-linking.

A good deal of research work has explored possible additions to the matrix that may aid both the speed of healing and the completeness of the final repair. Such additions have included growth factors to accelerate cell ingression and growth, and seeding of the matrix with cells to overcome the constraint placed on cell ingression rate by diffusion. Consideration must also be given to the promotion of revascularization. The ingression of new tissue will only occur if it can be supported with a nutrient supply, and seeding cells is useless if the support network cannot be regenerated within a short period of time.

The major variable remaining is the location of the repair, for which there exist two options: *in vivo* and *in vitro*. While all regenerated tissue must be incorporated into the body eventually, there are some repair procedures that can be initiated outside the body for reasons

of expediency. Needless to say, the choice for location of repair depends to a large extent on the nature of the tissue: some tissue types are better suited to *ex vivo* repair than others.

For example, it is common to culture epidermal tissue *ex vivo*, for a number of reasons:

- The epidermis is thin, allowing nutrients to be supplied to cells by the diffusion mechanism only
- The complexity of the epidermis is low, as there are few cell types and the dermis provides the necessary substrate for growth
- Vascularization is not required. It is not currently possible to stimulate angiogenesis outside the body.
- Integration into the wound site is relatively easy.

In vitro treatment is better suited to regeneration of the epidermis than of the dermis, as differentiation in the dermis is too complex to achieve with current technology.

However, the dermis is not suitable for regeneration *in vitro*. Vascularization is essential for complete integration of the dermis into the body. Currently, this is not possible outside the body. This means that dermal tissue cultured outside the body would degrade into scar tissue following transplantation, due to the absence of a nutrient supply.

For this reason, an *in vivo* procedure is to be adopted for dermal repair.

4.1 In vitro Vs. In vivo

(*The information in section 4.1 was taken from* Lanza *et al*, *Principles of Tissue Engineering*[3] *unless otherwise referenced*)

The following table outlines the advantages of growing tissue inside and outside of the body.

In vivo	***In vitro***
Many aspects of *in vivo* repair such as particular growth hormones cannot be replicated outside the body	Targeting of specific cells is possible (gene therapy for example) and risks for other cells within the body are minimised
In vivo repair is a lot less expensive than ex vivo repair considering the cost of growth factors, specialised labour and equipment	Recovery time can be decreased by simultaneous development of dermis inside the body and epidermis outside the body
Complete differentiation is possible including vascularization	Rapid growth is possible through the use of cytokines in concentrations which would be toxic within the body
Full integration into the body is simpler to achieve	Development of tissue from embryonic stem cells is possible, to make use of the rapid turnover in these cells
The body provides the correct physio-mechanical environment for structurally-adequate repair	Multiple experimental procedures can be tested outside the body, and the most suitable procedure can be selected

Table 4.1 Respective advantages of in vitro *and* in vivo *treatment techniques*

4.1.1 In vitro Treatment

Initially, design criteria must be established for the specific application for which the tissue is intended. Skin consists of multiple layers, and currently each layer must be cultured separately as cell differentiation outside the body is in its early stages (see section 4.6). Design criteria for skin equivalents are:

- Required dimensions (determined by the extent and depth of the damage)
- Structural and functional properties, such as:
  - The strength to function normally and resist cuts and abrasions
  - Temperature control (sweating or routing blood close to the surface for cooling)
  - Barrier to infection and to water loss
  - The elasticity to permit the normal range of movement
  - Aesthetic considerations dictate that the tissue should have the look and feel of natural skin.

The epidermis is particularly suited to *in vitro* regeneration by its dimensions. The supply of nutrients and oxygen to cells is limited by diffusional constraints, and the small thickness of the skin ensures that in general, growth is not limited by starvation. However, mixing of the growth medium is still required to maximise mass transfer, and this must be achieved without mechanically interfering with tissue growth. Stress during growth is desirable, however, and a tension/pressure cycle encourages the reinforcing of skin tissue so that adequate strength is obtained.

Growing the skin replacement on an extra-cellular matrix makes the skin simpler to handle when it comes time to transplant the skin to the wound. In addition, enzymatic treatment is not required to separate the skin from the bioreactor, so the dermo-epidermal junction is not disturbed. However, the presence of an ECM *in vitro* presents a limitation to the rate of mass transfer of nutrients and oxygen to cells in the matrix, and of metabolic waste products from cells in the matrix.

Finally, all tissue grown *in vitro* must at some stage be integrated into the body, and consideration must be given as to how this is to be achieved. Major factors include acceptance by the immune system, vascularization if required and the replacement of the graft by natural cells such that structural properties are maintained.

4.1.2 In vivo Treatment

It is important to note that the epidermal layer will regenerate spontaneously, but this will only occur if there is an adequate support network supplied by the dermal substrate underneath. The dermis does not regenerate if the damage is too great. To this end, *in vivo* treatment techniques for full thickness burns to date have concentrated on dermal repair.

In vivo repair techniques revolve around the implantation of a biodegradable extra-cellular matrix (ECM) to act as a scaffold onto which cells can attach during wound repair. An

advantage of *in vivo* treatment is that the wound is supplied with nutrients, cells and cytokines (growth factors) by the body, and all that is required is a matrix to guide the repair effort. The stability of the temperature and pH of the living environment provide good conditions for growth. Even though a precise knowledge of the chemical reactions and biological interactions which take place during wound repair is not required for *in vivo* repair, the original configuration must be known with reasonable accuracy so that the end goal is clearly defined. Design requirements for an *in vivo* skin regeneration template include:

- Interaction with components of the exudate supply to modify the kinetics which normally convert the exudate to scar tissue
- Easily accessible to migrating cells, including the adequate presence and distribution of macropores for cell and nutrient transport.
- Appropriate density, position and types of binding sites for cells must be present
- Biodegradable so that it can be removed from the wound site by the body after healing is complete
- Biocompatible so that the immune system does not reject it.

In addition to these design requirements, there exists the difficulty of choosing an appropriate performance measure by which success is defined. An example of such a measure is the moisture evaporation rate. However, there are many performance measures to choose from, and in many cases meeting one will mean failing to meet another.

The exudate is drawn into pores through mechanisms of capillarity and diffusion down the concentration gradient. It has been found that the optimal effective pore size lies between 20-125μm, as these pores are large enough for rapid transport of exudate, but small enough to provide sufficient sites for migrating cells to attach to[2]. Orientation of pore channels should be random, as for natural skin[3]. Porosity may be controlled in a number of ways, most of which involve manipulation of polymerisation conditions. An interesting variation was utilised by Butler *et al*[11]. A freeze-drying process under vacuum conditions was used to remove ice crystals by sublimation, leaving a highly porous matrix.

As noted above, one criterion for the suitability of the template is the biodegradability[3]. Degradation must occur with reasonable rapidity, but not so fast that adequate repair does not have a chance to take place. This degradation is ideally caused by lytic enzymes naturally present during wound repair, and should be complete enough that the products of the degradation (which must be non-toxic) can be transported from the site in the bloodstream. The time for complete degradation should be of the order of several weeks, and depends upon the severity of the wound. It is possible to use *in vitro* techniques to experiment with template characteristics prior to implantation to determine degradation rate data.

4.2 The Extra-Cellular Matrix

Primarily, the ECM provides a scaffold to which fibroblasts can attach. The extra-cellular matrix has been shown to be useful both as a carrier of cytokines and for quick closure of wounds[12].

When considering desirable properties for the ECM, it must be realised that the artificial ECM is replaced by fibroblasts, so the initial structure is refashioned to a large extent. This means that the synthesised ECM does not necessarily have to have the structure of natural skin. However, it has been found that repair is promoted by an ECM structure close to that of the original.

There exists a choice of materials from which the ECM can be created. Broadly speaking, these materials can be divided into natural and synthetic groupings. It is generally easier to control the properties of synthetics, and parameters such as strength, speed of degradation and permeability can be readily manipulated during production. Natural materials such as collagen have one decisive advantage however: they are usually easier for cells to stick to[13].

The ECM is typically constructed from a combination of collagen and GlycosAminoGlycans (GAGs). Collagen provides the template with structure and mechanical integrity, while the GAGs slow *in vivo* degradation and encourage correct biological activity. It has been suggested that hyaluronic acid, the largest GAG, acts as a transport mechanism for growth factors, releasing these when it is degraded.

4.2.1 Mechanical Considerations

In order to create a skin replacement with properties that approach those of the real thing, the physical requirements must be considered in addition to the biological requirements[14]. The affinity of the skin for the wound bed must exceed the affinity of air for the wound bed; otherwise pockets of air will build up in the interface. This is not only bad for healing; it also exposes the wound to potential sources of infection. In addition, the modulus of elasticity of the skin replacement must be close to that of natural skin so that the skin replacement can flex with the natural movement of the body without pulling away from the wound. Typically, once the modulus of elasticity is right (this is dictated by the material of construction) the desired flexibility is achieved through manipulation of the graft thickness.

The balance between growth and differentiation is affected by the hardness of the ECM[3]. Compliant gels promote differentiation, whereas stiffer gels support growth. Thus a gel that is initially hard and becomes softer as it degrades is desirable for the promotion of complete healing.

4.2.2 Cross-linking

Cross-linking of the collagen matrix is the primary means by which the mechanical properties and degradation rate of the matrix can be controlled. Cross-links stabilise the conformation of collagen fibres, and may be induced by chemical or physical methods. Natural collagen consists of polypeptide chains that form triple-stranded helical units. Cross-links between lysine residues mostly occur between the non-helical ends of the chains, and act to stabilize

the three-dimensional arrangement of these units. Unfortunately it is these non-helical ends that generate an immune response from the host. Once these ends have been cleaved from the chains, an alternative cross-linking method is required which links the helical regions of the collagen strands. Siegel[15] has patented such a method. After cleavage of the non-helical ends, cross-linking is induced through incubation with pyridoxal-5-phosphate in the presence of ionic copper or iron.

Glutaraldehyde (GTA), an efficient chemical agent, is the basis of the most widely used cross-linking method. Unfortunately, while GTA polymerises when in solution, with time GTA monomers are released, which are cytotoxic at concentrations as low as 10ppm. An alternative cross-linking method patented by Petite *et al*[16] involves the formation of amide bonds through the addition of diphenylphosphorylazide.

4.3 Growth Factors

There exist a number of growth factors that may be added to the ECM to stimulate healing. Specifically, the aspects of healing that should be assisted are vascularization and fibroblast growth. The requirement for angiogenesis results in a chicken-and-egg situation during wound healing. While fibroblasts are required to generate the physical support necessary for blood vessel ingrowth, these fibroblasts require a blood supply or they will not survive. This situation can lead to a petulant standoff, in which scar tissue is the only alternative. Through the use of growth factors, it is possible to stimulate both aspects of regeneration simultaneously.

Whilst growth factors are normally supplied to the wound site *in vivo* as part of the exudate, it is desirable to supplement the exudate with pure forms of these growth factors for a number of reasons:

- Chemically defined media reduce variability in experiments, and improve reproducibility.
- Research into growth factors allows partial regeneration of skin *in vitro* prior to transplantation.
- Biological fluids may be a source of toxins or viruses
- The growth factors may not be present in the quantities required for sufficiently rapid healing.

Growth factors known to promote fibroblast growth include interleukin, TGF-α and -β, bFGF and tumour necrosis factor α, along with many others. Unfortunately, while works exist which examine the effects of various growth factors, no direct comparison of growth factor types was located in the literature.

The main problem associated with growth factors is the delivery vector. Rapid diffusion combines with a short half-life to ensure that regular dosing is necessary in the absence of an adequate sustained-release mechanism.

4.3.1 Basic Fibroblast Growth Factor

Fibroblast growth factors have been shown to be modulators of cell proliferation, growth, differentiation and survival[17]. In particular, bFGF (otherwise known as FGF-2) has been shown to significantly inhibit wound contraction, particularly when applied in combination with a collagen matrix[12]. This combination was shown to be more effective than either bFGF or the matrix alone. In addition, bFGF stimulates the proliferation of fibroblasts, and promotes angiogenesis through accelerating the growth of capillary endothelial cells. Dosage has also been shown to affect healing: increasing the dose of bFGF was shown to improve both the healing rate and the success of the end result.

4.3.2 Acidified Fibroblast Growth Factor

Acidified fibroblast growth factor, FGF-1, has been demonstrated to stimulate revascularization, while inhibiting the formation of scar tissue[18]. As with many growth factors, repeated dosing is required for effective healing. FGF-1 can be expressed in recombinant *E. coli*, so large-scale production is not likely to present a problem.

In the study by Pandit *et al*., an FGF-1/collagen system was compared with both a control (no treatment) and a collagen scaffold only, and was found to produce better blood vessel regeneration than either of the other two cases. In addition, the FGF-1/collagen combination produced the strongest and most elastic of the regenerated skins, with the smallest inflammatory response during healing.

4.3.3 Keratinocyte Growth Factor

Keratinocyte Growth Factor is an important mediator of *in vivo* epidermal repair. KGF has been shown to selectively induce keratinocyte proliferation and differentiation[19]. It has also been shown to accelerate maturation of the dermo-epidermal junction, and to increase the thickness of the epithelium.

4.3.4 Transforming Growth Factor b

TGF-β inhibits inflammation while promoting angiogenesis (revascularization) and histogenesis[20]. A collagen sheet ECM impregnated with TGF-β was shown to stimulate the multiplication rate of fibroblasts. It has also been demonstrated to regulate epithelial-mesenchymal interactions. Until recently, a major disadvantage of TGF-β was the difficulty associated with obtaining and isolating it: a ton of bone yields just one therapeutic application of the growth factor. This patent[20] also covers the production of TGF-β through implantation of recombinant DNA into *E.coli* cells by means of plasmids.

4.3.5 Recombinant Human Growth Hormone

rHGH has been demonstrated to accelerate healing of burns through more rapid production of tissue to replace that which has been lost[21]. This finding is fairly generic, in that it applies to all types of wounds. There exists a concerning lack of specificity in this treatment, however, as the hormone affects all areas of the patient's body, and may lead to problems elsewhere in the patient.

4.4 Growth factor delivery vectors

Consideration should be given to how the chosen growth factor is to be delivered to the wound site. Many growth factors degrade rapidly within the body. A single large dose initially is not sufficient if the growth factor concentration is to be maintained at the wound site. One option is regular topical dosage, but this poses a number of problems. The wound must be disturbed in order to apply the dose, and it is not possible to achieve an even distribution of the growth factor throughout the wound site. The effects of a cyclic variation in growth factor concentration must also be investigated.

However, a number of alternative delivery vectors exist.

4.4.1 Delayed Release

An interesting delivery vector was explored by Kawai *et al*[22]. Gelatin microspheres were impregnated with bFGF and seeded into an acellular collagen matrix. This seeding was possible as the average microsphere diameter was smaller than the matrix pore size. The inconvenience of regular dosing was overcome by this sustained-release mechanism. Use of microspheres was seen to increase the growth factor retention time by a factor of about ten.

4.4.2 Gene Therapy

An area in which little work has been carried out to date is the application of gene therapy to dermal regeneration. It would be possible to genetically modify allogenic fibroblasts prior to seeding these in the ECM to overproduce a growth factor such as bFGF. This method would also eliminate the need for regular dosing. Gene therapy raises a number of significant concerns however. While the genetic modification itself is relatively straightforward, the primary difficulty is one of control. Unless the fibroblasts can be engineered so they do not pass the genetic modification on when they replicate, it is not possible to stop the growth factor production once treatment is complete, which will lead to unwanted side effects. Ethical issues associated with gene therapy must also be taken into account. It is for these reasons that gene therapy cannot be considered as a treatment option.

4.5 Seeding of the Collagen Matrix

4.5.1 Fibroblast Seeding

The presence of living fibroblasts in the dermal substitute means that vascularization is swift, and the wound heals more rapidly than if the matrix was acellular. Also, no growth factors explored promote the ingression of fibroblasts to the wound site, making seeding the preferred method to expedite repair.

Fibroblasts serve a number of roles in the regeneration of damaged skin. The first is the secretion and maintenance of components of the ECM that makes up the ‘backbone’ of the skin, ensuring that the general morphology of the skin is maintained even when cells die. It is only when this matrix is damaged that the skin has difficulty regenerating spontaneously. Secondly, they are an integral component of the support network for the epidermis, allowing

for physical attachment through the basement membrane, which lies along the dermo-epidermal junction. Support is also supplied by way of nutrients.

The reason for rejection of grafts by the immune system is the identification of that tissue as foreign. This identification is always based upon the surface molecules of the transplanted cells (as this is the only surface presented to the immune cells). It is important to realise that there exist two classes of antigens, class I and class II. While MHC class II antigens[23] are present in most cells (including keratinocytes), fibroblasts lack these surface molecules. This makes them, immunologically, relatively inert. This is fortunate from a tissue-engineering point of view, as dermal regeneration matrices can be seeded with allogenic fibroblasts without risking an immune response. Indeed, if allogenic fibroblasts are present, they will generally inhibit the penetration of the host's fibroblasts into the matrix[24].

The lack of an immunogenic response to allogenic fibroblasts opens up a number of exciting possibilities, one of which is the preparation of skin equivalent from volunteer donors, to be stored until needed[14]. Fibroblasts can typically be isolated from healthy skin through punch biopsies. This provides surgeons with a permanent skin replacement 'off-the-shelf'.

Approval from the appropriate regulatory bodies must be sought to seed the collagen matrix with allogenic human fibroblasts. While introducing foreign cells into the patient brings in the possibility for transfer of disease, previous work in the screening of blood donations and organs for transplant will hopefully minimise this risk.

Lamme *et al.*[25] examined the relationship between fibroblast seeding density and wound healing, using autologous fibroblasts. It was found that increasing the seeding density improved not only the healing time but also the cosmetic appearance of the final result. The time for which the fibroblasts were cultured in the matrix prior to transplantation was also found to have a large effect: increasing this time dramatically improved healing, as the fibroblasts had the opportunity to establish themselves within the ECM, laying down collagen and elastin fibrils.

4.5.2 Keratinocyte Seeding

Seeding the skin equivalent with a layer of keratinocytes greatly increases the rapidity with which the epidermal layer can be regenerated, given adequate support from the new dermal layer. However, the source of the keratinocytes must be considered. Allogenic keratinocytes are rejected by the immune system, so the keratinocytes seeded in the matrix must be drawn from the patient.

The activity of the keratinocytes, however, is a function of the location from whence they are removed. There is seen a decrease in proliferative potential as one moves from the basement membrane to the cornified layer of the epidermis. Indeed, when cells leave the basal cell layer they start down the path of terminal differentiation, and are thereafter limited to a maximum of about five cell divisions. The basal cells have the greatest ability to divide and grow: these are stem cells. Butler *et al.*[11] hypothesised that terminal differentiation of keratinocyte stem cells is triggered by the contact with other cells resulting from the

formation of a confluent layer of cells. It was demonstrated that when cultured to sub-confluence prior to harvesting, keratinocytes had colony-forming efficiencies as high as 80% (compared with less than 10% for uncultured keratinocytes). The keratinocytes thus harvested proved to regenerate epidermis much more rapidly than uncultured keratinocytes. The disadvantage of this technique is the time required for culturing. However, surgical treatment is often postponed for a number of weeks so that healing can be observed, which means that culturing of autologous keratinocytes is not out of the question.

4.6 Living Skin Equivalent

An exciting development in tissue regeneration is the culturing of a living skin equivalent (LSE) *in vitro*. The goal of LSE is the development of an entire living tissue *in vitro*, the idea being that, with the exception of nutrient and oxygen supply, the tissue is self-sufficient. To this end, researchers attempt to seed the matrix with the potential for differentiation into all cell types required for functional skin. This potential is found in stem cells, a popular source of which in recent research has been human foreskins.

A major difficulty associated with the growth of a dermal equivalent *in vitro* is that of contraction of the matrix. Once they have established themselves within the matrix, connective cells such as fibroblasts pull collagen fibrils towards themselves, which results in an overall reduction in the volume of the matrix. This tendency has been overcome through the development of anchoring techniques that fix the length and breadth of the matrix, allowing contraction in the thickness dimension only[26]. A second problem is the time required to culture such an equivalent from autologous cells, as allogenic keratinocytes will be rejected by the immune system.

These limitations mean that thus far, the main use for living skin equivalent is as a testing vehicle for new treatments. Applications outside skin regeneration include assessing the effectiveness of sunscreens, and also in investigating the diffusion rate of drugs through skin.

5 REGENERATION STRATEGY

The strategy believed to be best suited to the task is an in vivo *approach, involving the implantation of a collagen ECM seeded with cultured allogenic fibroblasts. Gelatin microspheres are to be utilised for the delivery of basic fibroblast growth factor to the wound site.*

5.1 Key Parameters

5.1.1 In Vivo

The repair is to occur *in vivo*, mainly to facilitate integration into the body. Vascularization is an aspect of integration that is essential for regeneration, and cannot be replicated *in vitro*. *In vivo* repair has the additional advantage over *ex vivo* of low cost, both in terms of materials and labour. The *in vivo* approach also reduces the trauma associated with multiple surgical procedures. For *in vitro* methods, multiple procedures are dictated by the need to protect the wound site while a more permanent solution is created outside the body.

5.1.2 Extra-cellular matrix

The matrix is to be constructed primarily from type I collagen and glycosaminoglycans (GAGs). This material was deemed to be most appropriate as it is possesses the required properties, and is relatively inexpensive. In addition, collagen is the main material from which the natural extra-cellular matrix is constructed. Collagen is not the only constituent of the natural ECM. However, the implanted matrix exists to provide a template for tissue regeneration and to prevent wound contraction, tasks that are fulfilled by collagen alone. During the repair process, the matrix is entirely replaced by the body as the fibroblasts establish themselves.

The ECM is to be prepared through polymerisation of a solution containing approximately 6mg/mL of bovine collagen and 2mg/mL elastin at 37°C[25]. Implementing the freeze-drying technique used by Butler *et al*[11] can increase the porosity of the matrix. Ideally, the mean pore size in the matrix is to be 80μm[25].

Cross-linking of the matrix is to be achieved with glutaraldehyde. Further work is required, however, to establish the cytotoxicity of the products of degradation of GTA. The extent of cross-linking controls the rate of degradation. Residence time is to be approximately two weeks, but this depends on the severity of the wound. The matrix should be firm initially to promote growth, becoming more compliant as the cross-links break down. A softer matrix encourages differentiation and maturation, to give a final product closer to natural skin.

In the style of Integra™, an outer silicon layer 0.3mm thick is to provide protection for the wound and control moisture loss while the dermis establishes itself. While the dermal layer is re-establishing itself, autologous epidermal tissue can be cultured *in vitro*, so that it can be applied as soon as the silicon sheet is removed.

5.1.3 Growth factors

The growth factor chosen to promote healing and revascularization is basic fibroblast growth factor. bFGF has also been shown to reduce the rate of wound contraction, which is desirable from the point of view of minimising scarring. Porous gelatin microspheres are to be utilised for the controlled delivery of the growth factor[22]. The advantages of this delivery vector include

- Delayed release of bFGF, which maintains the concentration of growth factor at the wound site and removes the need for regular dosing. Controlled release is desirable to counter the rapid degradation of bFGF *in vivo*, and diffusion away from the wound.
- Ensures efficient use of growth factor, reducing the amount of bFGF required.
- Prevents bFGF from causing unwanted effects elsewhere in the patient

The mean diameter of these microspheres is to be approximately 40μm. For the method of microsphere production, refer to Kawai *et al*[22]. As this is smaller than the mean ECM pore size, these microspheres should disperse evenly within the matrix

5.1.4 Fibroblast seeding

The ECM is to be seeded with allogenic fibroblasts. Advantages of allogenic cells include

- Due to the low immunogenicity of fibroblasts, the matrix can be prepared for instant application when required, so multiple surgical procedures are not necessary
- Further trauma is not inflicted on the patient by extraction of autologous fibroblasts
- Fibroblasts can be cultured in the matrix to establish themselves prior to application

The culturing of fibroblasts also reduces wound contraction through lessening their tendency to differentiate into their contractile phenotype, the myofibroblast[25]. Fibroblasts are to be collected from volunteer donors by means of punch biopsies, and seeded into the matrix at a density of approximately 5×10^5 cells/cm^3. Following seeding, the cellular matrix is to be cultured in the matrix for a minimum of ten days where possible to allow the fibroblasts to establish themselves. The addition of ascorbic acid will promote the deposition of ECM components *in vitro*[25].

5.1.5 Surgical procedure

Implantation of the matrix into the patient involves the following steps

- Excision of wound. The removal of necrotic tissue from the wound bed is desirable in order to prevent infection and allow room for tissue regeneration
- Implantation of ECM at wound site, held in position by elastic bandages. The ECM will not be strong enough to hold sutures. As a result, the patient should be kept still to minimise stresses on the wound.
- Following establishment of the dermis, the silicon sheet is removed. An autograft can then be applied to assist epidermal regeneration, cultured outside the body if required.

6 CONCLUSIONS AND RECOMMENDATIONS

Advances in the field of burns treatment have great potential both to speed recovery time and to reduce suffering. The various components that make up this regeneration strategy have all been demonstrated to work previously, so it is anticipated that no major problems will be encountered in the engineering of this solution.

6.1 Recommendations for future work

As this solution is entirely based upon review of available literature, a great deal of experimental work is required before it can be implemented. Experimental work should concentrate on:

- Growth factors. A direct comparison of the effectiveness of the growth factors available should be conducted. Parameters to be quantified should include:
  - Degree of differentiation
  - Rate of growth
  - Extent of angiogenesis
  - Degree of wound contraction
- Storage conditions. If this skin equivalent is to be available to surgeons as an 'off-the-shelf' solution, it is desirable to determine the following characteristics.
  - Optimum storage conditions
    - Freezing
    - Low temperature
    - Under solvent solution
  - Length of time fibroblasts will remain viable
  - How storage will affect growth factor
  - Whether gelatin microspheres will retain functionality during storage
- Cross-linking
  - Comparison of cross-linking methods
  - Cytotoxicity of degradation products
  - Effect on biodegradability

With serious burns as common as they are (although most are preventable), the field of tissue engineering has much to contribute to this area of human injury. Research has demonstrated that better methods for the assistance of skin regeneration do exist, and further work is essential so that these may be applied in burns treatment centres as soon as possible.

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