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Review

New technologies for burn wound closure and healing—Review of the literature

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Abstract

Methods for handling burn wounds have changed in recent decades. Increasingly, aggressive surgical approach with early tangential excision and wound closure is being applied leading to improvement in mortality rates of burn victims. Autografts from uninjured skin remain the mainstay of treatment. Autologous skin graft, however, has limited availability and is associated with additional morbidity and scarring. Severe burn patients invariably lack sufficient adequate skin donor sites requiring alternative methods of skin replacement. The present review summarizes available replacement technologies.

Keywords: Burn wound; Wound healing; Allograft; Keratinocyte culture; Tissue engeneering

Contents

1. Introduction

Severe burn injuries cause extensive damage and are notoriously complicated by loss of body fluids. More often than not, such wounds become seriously infected further aggravating morbidity. Despite advances in burn management, the mortality rate of these injuries continues to be high and the search for economical and easily available topical measures to control burn wound infection continues [1]. Invariably, many of the different methods applied for local treatment are still controversial [2]. Irrespectively, the main requirement in burn wound management is an economical, easy to apply, readily available dressing or method of coverage that will provide good pain relief, protect the wound from infection, promote healing, prevent heat and fluid loss, be elastic and non-antigenic and adhere well to the wound

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[2,3] while waiting for spontaneous epithelialization of superficial partial thickness burns or for permanent coverage with autologous epithelium of deeper burn wounds.

Methods for handling burn wounds have changed in recent decades. Increasingly, aggressive surgical approach with early tangential excision and wound closure is being applied. It is probably the most significant change in recent years leading to improvement in mortality rates of burn victims at a substantially lower cost [1,4–12]. By shortening hospital stay, early wound closure reduces pain associated with local burn wound care, number of operative procedures and infective complications. It also decreases the severity of hypertrophic scarring, joint contractures and stiffness, and promotes quicker rehabilitation [4,6]. Irrespective of any other consideration, early healing is paramount for good aesthetic and functional recovery. It has been clearly demonstrated that disruption of epidermal–mesenchymal communication due to a delay in epithelialization, increases the frequency of developing fibrotic conditions [13] such as scar hypertrophy and contractures.

Autografts from uninjured skin remain the mainstay of treatment for many patients and skin graft preservation for the purpose of delayed application is still a basic tool in burn treatment and plastic and reconstructive surgery [14]. Autologous skin, however, has limited availability and is associated with additional scarring [1]. Severe burn patients invariably lack sufficient adequate skin donor sites [15–20] necessitating alternatives for burn wound coverage and healing. Additional limitation to the modality of autologous partial thickness skin grafting is the creation of additional donor site wounds equivalent to second degree burns thus further increasing the total body surface area (TBSA) affected [4].

2. Amniotic membrane

Severe burns, unfortunately, continue to present a major challenge to the scarce medical resources available particularly in developing countries [3]. Though the primary objective remains excision of dead tissue and restoration of skin continuity without delay, topical burn wound therapy and temporary coverage remain an essential part of the overall burn therapy scheme [4]. Since 1910, allogenic amnion has been used as a biological wound dressing [21]. It has been claimed that it is one of the most effective biological dressings ever used in burn treatment [1,21–23]. In fact, efficiency of amniotic membrane in preserving healthy excised wound bed and maintaining low bacterial count in contaminated wounds parallels that of human skin allograft dressings. Contrary to human skin allograft, it has a fragile structure and is technically more difficult to handle [24]. Amniotic membrane is a thin semi-transparent tissue forming the innermost layer of the fetal membrane. It has an avascular stroma and a thick continuous basement membrane with a full complement of collagen types IV and V and laminin, and contains several proteinase inhibitors [25,26]. Its use has special appeal in developing countries particularly where religious barriers preclude the acceptance of bovine and porcine skin [1] or cadaveric skin. Advantages of human amniotic membranes in burn wound management such as reducing loss of protein, electrolytes, fluids and energy as well as reducing risk of infection and antibiotic administration, avoiding bulky dressings and minimizing pain and analgesia associated with dressing changes and accelerating epithelial regeneration reducing length of hospitalization have all been well documented in the medical literature [1,21,27–29].

Placental membranes whether harvested following normal vaginal delivery or caesarian section are invariably contaminated. There is, however, a greater risk of contamination from pathogenic bacteria on placentas from vaginal deliveries [30]. Serologic tests at the moment of the childbirth and six months later, is a good tool to safeguard against the possibility of transmitting infectious diseases such as syphilis, auto-immune deficiency syndrome (AIDS) and hepatitis B and C viruses [1]. Washings with antibiotic solutions, lyophilization, sterilization with 60Co gamma radiation, or long-term glycerol preservation [1,26,28,31] have all been used to free amniotic membranes from bacterial and fungal contamination. Amnion used as a biologic dressing has been proven to be impervious to micro-organisms and is usually free from toxic material [21] however, disease transmission remains a possibility as with any biological material. Human cytomegalovirus (CMV), known to replicate in vitro in human fibroblastic cells, was found to replicate in epithelial human amnion (HA) cells as well [32] and may constitute a real risk whenever amnion is used as a biologic dressing. This is more of a theoretic concern than a real threat. In a study performed on severely burned immunosuppressed patients 52% became infected with either herpes simplex virus (HSV) or CMV or both with no significant association of such infections with mortality [33]. In another retrospective survey of serum for viral antibodies in pediatric burn patients, in 33% of the children CMV infection developed; in 25% herpes simplex infection; and in 17% adenovirus infection. In all of the most severely burned children CMV infections developed, and both primary and reactivation infections were observed [34]. With these facts, any potential risk of amniotic membranes or any other biologic material transmitting CMV or herpes simplex infection becomes irrelevant. A real concern for infection, however, remains. Unlike skin allografts, amniotic membranes do not get incorporated and vascularized [24]. Bacterial growth invariably occurs underneath the membrane with time. Though amniotic membranes allow rapid epithelialization and early healing in superficial and intermediate depth dermal burns, in deep dermal burns the membrane is not adequate and usually disintegrates before healing occurs [35]. Human amniotic membrane incorporating 0.5% silver nitrate has been described with claimed better therapeutic effect than plain membranes [23].

Allogenic amniotic membranes are collected from the placentae of selected and screened donors [36]. The membranes can be used fresh after processing by washing successively in sterile saline, 0.05% sodium hypochlorite solution and sterile distilled water until they are completely cleared of blood particles. They are then sterilized by gamma irradiation at 25 kGy [36]. Preservation of allogenic amnion is possible with glycerol. Neither the treatment with glycerol, nor the pre-transplantation rehydration destroys the surface monolayer amniotic epithelium. Its complex architecture remains intact during the preservation process [21]. Human amnion allografts can also be preserved by lyophilization or deep-freezing and subsequent radiation sterilization with a dose of 35 kGy [22]. Lyophilised, irradiated, human amnion is a temporary biological dressing conveniently available off-the-shelf [1,36]. A method of freezing and sterilization has also been developed enabling human amniotic membranes to be stored at −60 °C for more than six months [37]. Sterilization with 60Co gamma radiation eradicates bacteria and fungi. Transmission of syphilis, AIDS, hepatitis B and C viruses can be effectively checked with systematic serologic tests at the moment of membrane harvesting and six months later before clinical usage of the preserved membranes [28]. However, similar to any other biologic material, risk of disease transmission, though minimal, can never be totally precluded.

Human amnion is primarily used to cover debrided second degree burns until complete healing, which varies depending upon the extent and depth of the wound and the amount of exudates, is achieved [1,21,27]. It may also be used as a temporary coverage of full thickness wounds preparing the wound bed for eventual necessary skin transplantation [2]. Human amniotic membranes can provide a useful cover for microskin grafts as well or an overlay of widely meshed autografts promoting early epithelialization and rapid wound healing [38,39].

3. Human cadaver allografts and xenografts

Whenever available skin donor sites are limited or when the overall patient condition does not permit immediate coverage of excised burn wounds with autologous skin, there may still be a clinical need for human cadaver allograft skin (HCAS) for a temporary biologic dressing [20,40,41]. Similar to allogenic amnion, HCAS may also be used as a dressing to cover widely meshed autografts in extensive burns [42]. It must be noted though that the usage of allograft skin has been associated with slightly increased number of operative procedures per percent of TBSA burn [43]. As the demand for skin allografts has increased, and to ensure a supply of good quality material, the responsibility for processing, storage and evaluation of graft performance of preserved skin has become an important issue of banking organizations [14] particularly when cell viability in allograft skin may be an essential consideration for clinical repair of wounds whenever cytokine activity or dermal integration are desirable [44].

Nonetheless, serious problems are associated with HCAS including limited supply, variable and occasionally poor quality, inconvenience of harvesting skin in the mortuary and ultimate immune rejection [40,42]. The availability of cadaveric allograft is also often limited by potentially pathogenic microbial organisms [45]. Despite strict adherence to American Association of Tissue Banks (AATB) protocols or other similar protocols potential pathogenic microbial and viral contamination of cadaveric allograft skin does not reach zero [42,45,46]. Five percent of harvested cadaveric skin is usually discarded due to positive cultures [45]. Methicillin resistant Staphylococcus epidermidis, (MRSE), is the most predominant organism (22.2%), followed by Gram-negative rods as a group (18.5%), with Aspergillus species being the least predominant isolate [45]. Moreover, possible transmission of viral diseases such as auto-immune deficiency syndrome is a serious problem that has led some authorities to prohibit the usage of HCAS under any consideration even though some claim that the risk of HIV transmission is not a drawback for the use of glycerolized skin [47].

After harvesting and just prior to further tissue bank processing, human cadaver skin grafts exhibit approximately 60% of the metabolic activity found in fresh skin samples obtained from living surgical donors. If allowed an overnight (18–24 h) incubation period at 37 °C, cadaver samples show a recovery of their metabolic activity to 95% [48]. When stored in liquid media at 4 °C, cellular metabolic activity of the cadaver skin declines steadily, arriving at a measurement below that of cryopreserved skin in less than five days storage [48]. At present, in some centers, cadaveric skin is processed and preserved by glycerolisation or otherwise and is exceptionally used as fresh in comparison to 1973 when skin was stored frozen at −28 °C [49].

Various preservation methods of non-viable skin allografts with maintenance of structural integrity have been developed [50]. After initial disinfection using a cocktail of antibiotics, cadaveric skin can be cryopreserved [51]. Unfortunately antibiotic disinfection procedure fails to decontaminate a significant proportion of allografts [51]. Contaminated grafts may still be salvaged after thawing by peracetic acid (PAA) disinfection combined with immersion in high concentrations of either glycerol or propylene glycol [51]. Cadaveric skin stored frozen at −28 °C provides graft material with good cellular viability comparable to that of fresh skin stored at 4 °C for four days [44,49]. Graft performance of cryopreserved skin can be maintained at a relatively good level for a period of five years; however, it decreases sharply thereafter [14]. To avoid the frequent problems encountered with the use of fresh cadaver skin and the expenses of cryopreservation, HCSA is now mostly processed and preserved by glycerolisation [49]. The low costs of glycerol preserved allograft (GPA) skin banks have gained rapid popularity [19,42]. High concentration glycerol

dehydrates the skin by osmosis and diffusion out of the cells and skin matrix, respectively preventing or limiting the many degradation reactions that can occur within stored tissues including enzymatic digestion, oxidation (peroxidation) and hydrolytic reactions, as well as minimizing the detrimental effects of microbial growth [50,52]. The considerably easier handling and storage of glycerol preserved allograft skin makes it preferable to cryopreserved allografts [43] however, GPA provides no viable coverage material and lacks the beneficial effects of integration and vascularization of viable allogenic grafts. One of its main benefits though is the need for less frequent change of the allograft compared to several years ago due to the effects of glycerol in decreasing antigenicity of the skin [49]. Similar to other biologic dressings, GPA is mainly used as a temporary cover on freshly excised wounds or as an overlay on widely expanded autografts. It is used also to improve the quality of the wound bed prior to autografting with cultured keratinocyte sheets [19,20]. The usage of cryopreserved skin, however, is still recommended in cases where the integration of a dermal component as a permanent part of wound closure is desired [53]. Allograft dermis has been shown to be incorporated over time without rejection. Subsequently, dermal allografts may be prepared separately in a lyophilized form and can be used successfuly in association with epidermal autografts [20].

On the other hand, xenografts have been used for hundreds of years as temporary replacement for skin loss. Donor species include frog, lizard, rabbit, dog and pig. Although these grafts provide a biologically active dermal matrix, the immunologic disparities prevent engraftment and predetermine rejection over time [20]. It must be stressed that xenografts and allografts, are only a means of temporary burn wound cover. True closure is achieved only with living autografts or isografts (identical twins) [53]. One exception, though, is human skin allografts in patients taking the usual dosages of immunosuppressants for renal transplantation. In such category of patients, skin allografts seem to survive indefinitely with minimal repopulation of skin allografts by autogenous keratinocytes (KC) and fibroblasts. In case of discontinuation of immunosuppression, the skin allograft does not reject acutely. It persists clinically and the allograft cells are destroyed and replaced slowly with autogenous cells [16].

Vascularization of viable syngeneic, allogeneic and xenogeneic skin grafts starts to get established 3–4 days after grafting [54]. By quantitative determination of graft vascularization using radioactivity determination following host injection with labeled red blood cells, for any one donor–host combination, full-thickness grafts demonstrate relatively more blood than split-thickness grafts, presumably because of large vessels in the deep part of the dermis in full-thickness grafts that become connected to the host vascular channels [54]. Vascularization of non-mammalian xenografts is significantly inferior. This is more plausibly explained on the basis of defective self recognition than as representing a reaction to foreign determinants [54]. It can be demonstrated also that grafted allograft skin becomes relatively more irrigated by blood than the corresponding normal skin [54]. On the other hand, reconstituted freeze dried allogeneic skin grafts exhibit virtually no blood flow, a phenomenon possibly analogous to the ‘no reflow’ phenomenon of microsurgery [54].

By its adherence to the wound bed, viable human cadaver allograft is believed to decrease or control microbial wound contamination to a sufficiently low microbial count to allow successful application of autograft [55]. Take and vascularization of allograft, however, does not guarantee that a wound bed is free of microbial contamination [55]. If the beneficial effects of temporary skin grafts do indeed depend on their capacity to become vascularized, fresh skin appears preferable to reconstituted freeze dried skin [54]. Histological, histochemical, ultrastructural, and radiolabeling characteristics of the microvasculature in regional nodes draining skin allograft sites show a rapid rise in lymphocyte migration indices and the apparent plugging of intermediate sinuses by lymphocytes suggesting that both increased entry and decreased egress of recirculating cells contribute in ‘‘lymphocyte trapping’’ [56]. These results do not necessarily indicate that one type of graft is better than another in clinical practice [54].

4. Keratinocyte culture

Cell therapy is an emerging therapeutic strategy aimed at replacing or repairing severely damaged tissues with cultured cells [57]. The culture and transplantation of keratinocytes are definitely a major and important progress in the treatment of severe burns [58]. Epidermal regeneration obtained with autologous cultured keratinocytes (cultured epithelial autografts (CEAs)) can be life-saving for patients suffering from massive full-thickness burns [59,60]. In 1975, serial subculture of human keratinocytes was first described. Clinical application of this discovery was made possible after the preparation of these cells into epithelial sheets. In 1981, the earliest application of cultured autologous epithelia was made for the treatment of extensive third degree burns. Cultured epithelia avoid the mesh aspect obtained with a split-thickness autograft as well as the discomfort of skin graft harvesting for the patient [61] however, there is a time delay of 2–5 weeks for culture of the autologous sheets of keratinocytes which is a major handicap to the technique [62–64]. More recently, an automated membrane bioreactor was developed to produce on a large scale cultured skin grafts at significantly reduced cost and time of transplantation down to two weeks time. The computer uses the obtained information to control medium change and to predict the end of cultivation largely eliminating the risk of human error. The computer controlled reactor is modular, allowing the production of up to 0.5 m^2 of keratinicyte culture sheets at one time [65].

The most important advantage of cultured keratinocyte allografts is the large surface area obtained from a relatively small biopsy of healthy skin from the patient. A major disadvantage, nevertheless, is the delay, which is too long, necessary to provide cultured keratinocyte sheets for clinical application [61]. Fragility and difficult handling of the grafts, an unpredictable ''take'' and extremely high costs are other major disadvantages [59]. Storage and preservation of viable sheets have also been a major handicap [66]. Enzymatic detachment of the confluent multi-layered keratinocyte sheet from the irradiated fibroblast feeder layer which is a critical step of the classical culture method is another factor affecting success of the technique and may be largely responsible of the unpredictable clinical graft take. This critical step of the technique leads to a temporary loss of α6β4-Integrins essential for cellular adhesion [20,57]. Recently described membranes based on blends of poly(-ether imide) (PEI) with poly(benzimidazole) (PBI) could provide a tissue compatible scaffold with lowered adhesive properties, and might be a useful tool for the transfer of cells by passing enzymatic detachment [67]. A recent study on the growth of keratinocytes at the culture medium/air interface has led to the identification of a novel thin sheet-like matrix that supports adherent cells. This novel matrix consists of components secreted by keratinocytes, including type IV collagen, and laminins 1 and 5, that self-assembled to a membrane structure. Detailed features of the membrane strikingly resembles those of the basement membrane in vivo [68]. Clinical application and value of this finding remains yet to be determined.

Widespread use of cultured autografts has been primarily hampered by poor long-term clinical results that have been consistently reported by different burn units treating deep burns, even when cells were applied on properly prepared wound beds [20,57,69]. Though CEAs represent the common standard in clinically applied tissue engineered skin substitutes and do constitute viable and durable cutaneous epithelial coverage, they are unsuitable as a permanent skin substitute in burn patients with major full thickness burns [20,57] because they lack the essential dermal component. Dermal substitution in association with CEAs is required to enhance results [57]. Nevertheless, deep second degree burns remain an application of choice for the cultured epithelia, as the presence of dermis limits retractions responsible for functional complications usually observed in third degree burns where dermis is absent [61].

With due attention to safety and security, a bank of allogenic keratinocytes has been created to provide readily available sheets for immediate clinical application [61]. Although cultured keratinocyte allografts are initially accepted, grafted donor cells are gradually replaced by recipient elements. The precise mechanisms underlying this process are not clear [70]. Keratinocyte stem cells (KSCs) could act as antigen presenting cells thus explaining ultimate rejection of allogeneic cultured epithelium [71]. Cultured allogenic keratinocytes have been described in the treatment of leg ulcers, repair of skin donor site harvested for split-thickness autograft, and in second degree burns with encouraging results. Allogenic keratinocytes application is considered as the first phase of treatment of extensive deep second degree burns while awaiting autologous cultured keratinocytes [61]. The cost effectiveness of this modality as compared to GPAs still needs to be determined.

Chimeric xenogeneic–syngeneic graftable sheets consisting of histologically well-organized epidermis presenting basal and suprabasal cell layers previously obtained in vitro have also been developed and tested experimentally with good graft take. Cell phenotyping after healing revealed the presence of only syngeneic keratinocytes, whereas xenogeneic cells were passively eliminated without a total rejection of the chimeric implant. This selective and passive elimination of xenogeneic keratinocytes proceeds through cellular and humoral immunity activation. Data suggest that this chimeric culture method can be used for cutaneous therapies such as large congenital nevi, skin ulcers, and extensively burned skin. Moreover, since the ultimate aim in allogeneic and xenogeneic transplantation is to achieve an immunological acceptance and tolerance to these foreign tissues, the chimeric culture approach may provide ways to lighten tolerance phenomena on cutaneous tissue [64].

5. Tissue engineering—bilayered substitutes

Numerous problems have been encountered with regular keratinocyte cultures based on the original technique described by Rheinwald and Green [72] particularly when using mass-produced complex media. New keratinocyte culture technologies and/or new ''delivery systems'' have been developed to overcome these problems [70]. To circumvent the enzymatic step during the standard cultivation procedure and to simplify handling, keratinocyte cultivation is at present combined with various natural or synthetic carrier materials like polyurethane membrane, silicon-collagen membranes, hyaluronic acid-based (HA) membranes, collagen sponges, and fibrin glue [57]. It is also now clearly evident that success of cell therapy with high reproducible keratinocyte ''take'' and with permanent clinical results, requires cultivation and transplantation of stem cells, subconfluent noncontact-inhibited cells, with higher proliferative and wound healing capacity, rather than confluent cell layers where cellular differentiation has been stimulated [57,69]. Despite all these advancements and the numerous delivery systems that have been reported, most studies are limited to animal wound bed models. There are a few small clinical studies that have demonstrated enhanced healing using mainly subjective methods. There is a need for controlled, randomized clinical trials to prove the efficacy of keratinocyte delivery systems [73]. The best technique to deliver cultivated autologous keratinocytes with optimal growth potential after the shortest possible cultivation period and how can this transplantation technique be combined

with a dermal substitute is still an incompletely resolved question [74].

The application of tissue engineering technology to wound healing has resulted in the development of a number of ''living skin equivalents'' progressively shifting from autologous grafts to bioengineered grafts [20]. These in general are a combination of living cells and a supporting matrix [75]. Practical and safe transplantation necessitates ''easy to handle'' scaffolds that could be fabricated as carriers for the transfer of not only epithelial cells but of dermal elements as well. Application of these principles led to the revolutionary construction in vitro before grafting of artificial bilayered skin composed of cultured auto-keratinocytes on allo-dermis obtained from fibroblasts (FB) cultured in a specially designed scaffold [20,57,58,69]. An example of such a scaffold suitable for skin tissue engineering is an absorbable chitosan–gelatin asymmetric bilaminar structure, recently described, fabricated by freezing and lyophilizing methods. Keratinocytes can be co-cultured with fibroblasts in this scaffold to construct an artificial bilayer skin in vitro [76]. The new keratinocyte culture system on a dermal equivalent or substitute suitable for skin wound closure is flexible and has good mechanical properties resulting in good graft take without rejection [58,77]. Living bilayered skin construct (BSC), consisting of human neonatal keratinocytes and fibroblasts in a collagen matrix and laboratory-grown bilayered living skin substitute (LSS) have already been tested clinically to successfully treat a variety of wounds [78,79]. Numerous issues still remain to be resolved before this treatment modality becomes widely accepted and before it becomes applicable to cover extensive burn wounds.

Unique among skin tissue engineering technology is the Tissue Tech autograft system (Fidia Advanced Biopolymers S.r.l., Padua, Italy), as it incorporates an autologous dermal substitute and an autologous epidermal replacement, autograft. Each includes a matrix of a hyaluronic acid ester to promote cellular migration and graft take [80]. As a result of extensive research, innovative biodegradable matrices for cell delivery have been developed based on an ester of the naturally occurring extra cellular matrix (ECM) molecule, hyaluronic acid. With this system both autologous dermis and autologous epidermis can be produced which will be completely integrated when grafted. Hyaff-NW (Fidia Advanced Biopolymers S.r.l., Padua, Italy) is a 100% benzyl-esterified derivative of hyaluronic acid processed into fibers and prepared as a flat, non-woven pad dressing, which can be seeded with autologous fibroblasts before grafting [81]. Laserskin (Fidia Advanced Biopolymers S.r.l., Padua, Italy) is also a 100% benzyl-esterified derivative of hyaluronic acid made into a specially designed membrane for the culture and delivery of autologous keratinocytes. Laser-drilled microperforations allow keratinocytes to migrate through the membrane into the wound bed [72]. This technology has led to the development of the composite biocompatible skin graft (CBSG) also refered to as composite biocompatible epidermal graft (CBEG), a composite laserskin graft (CLSG) consisting of autologous keratinocytes cultivated on a pliable hyaluronate-derived membrane (laserskin) that has been pre-seeded with autologous/allogenic dermal fibroblasts. Basement membrane proteins of CBSG are protected from the dispase treatment (a necessary step in the standard cultured epithelial autograft technique) since keratinocytes are directly seeded onto laserskin [82–84]. The CBSGs are much easier to handle than the conventional cultured epidermal autografts and are good human skin substitutes in terms of durability, biocompatibility, high seeding efficacy for keratinocytes, high graft take rate, and low infection rate [83,84].

Transplantation of cultured autologous keratinocytes as a single-cell suspension in a fibrin glue matrix combined with allogenic skin grafting is also being investigated [57,59]. Apligraf (Organogenesis, Canton, MD) (type 1 bovine collagen with cultured human fibroblasts and keratinocytes) and Epicell CEA (Genzyme, Boston, MD) (auto keratinocytes and fibroblasts cultured separately then combined on a collagen–glycosaminoglycan matrix) are commercially available products [20]. Though results are encouraging, their use, however, is still limited to certain selected conditions.

6. Cultured dermal substitutes—fibroblast cultures

An in vivo study of cultured artificial dermal substitutes showed that an artificial dermis containing autologous cultured fibroblasts enhances more the re-epithelialization of a full-thickness skin defect when compared to an acellular dermal substitute scaffold [85] stressing the importance of incorporating fibroblasts in any bioengineered construct for skin replacement and healing. Allogeneic cultured dermal substitute (CDS) (artificial skin) can be prepared by culturing fibroblasts on a specially designed scaffold such as two-layered spongy matrix of hyaluronic acid and atelo-collagen (Col) [86–88]. A biodegradable salt-leached porous gelatinous scaffold is also appropriate to seed cultured fibroblasts. It has been demonstrated that the fibroblasts cultured under such conditions become mainly attached on the surface of the pores in the scaffold, whereas cells seeded on freeze-dried scaffolds are only widely distributed and aggregated on the top and the bottom of the scaffold. After 14 days of culturing, the fibroblasts exhibit a good affinity to, and proliferation on, the gelatin scaffolds without showing any signs of biodegradation [85].

CDS can be cryopreserved and transported to other hospitals in a frozen state retaining its ability to release essential cytokines for wound healing particularly vascular endothelial growth factor (VEGF) [89]. Cryopreserved allogeneic CDS functions as an excellent cell therapy for intractable skin ulcers as well as for burns and other skin defects [87,88]. The wound surface, however, must be

checked rigorously for the occurrence of infection during the healing process to guarantee a favorable outcome [86]. Taking into account the manufacturing cost, coupled with the potency of VEGF release, a two-layered sponge of HA and Col with a weight ratio of 5/2 is very promising for commercial application [89].

Various artificial skin substitutes are available commercially. They are effective for management of contractures, chronic wounds, and chronic skin illnesses. They may decrease or avoid the risk of donor area morbidity, which is more difficult to treat in children, as they can be used in conjunction with autologous cultured epithelium applications [90]. Decellularization of porcine skin to produce an acellular dermal matrix (ADM) for possible biomedical applications has also been described [91]. The practical application of ADM in the management of burn wounds still needs to be clarified.

Autologous CDS, on the other hand, allow quick wound bed preparation that would take a thin split-thickness autologous skin graft. Clinical trials with this therapeutic modality have yielded extremely encouraging results without development of severe contractures, expected following very thin split-thickness skin grafts (STSG), over a period of several months. The application of autologous CDS is promising for the treatment of extensive burn scar contractures particularly in children [92].

7. Organotypic skin-equivalent cultures

Biomedical science has made major advances in understanding how cells grow into functioning tissue. The signaling mechanisms used to achieve this are slowly being dissected. Tissue engineering is the application of that knowledge to the building or repairing of organs, including skin, the largest organ in the body [95]. Culture of human keratinocytes and fibroblasts has opened new avenues in the study of skin biology [93,94]. It has been demonstrated that preservation of the basic epithelial–mesenchymal interactions allows for highly complex ex vivo function of epidermal cells [95]. Fibroblast and keratinocyte interaction modulate the levels of MMP-2 and -9 and their inhibitors produced by these cells. This interaction may be critical for a better healing quality at a late stage of the wound healing process [13]. The new approach taken in tissue engineering is based on the preparation of organ fragments that preserve the basic epithelial/mesenchymal interactions but also ensure appropriate diffusion of nutrients and gases to all cells [95]. Such fragments allow primary cells ex vivo to preserve most of the functional attributes of the in vivo system. Clearly, the effect of the extracellular matrix is critical in this system in order for the cells to proliferate and differentiate ex vivo [95]. Moreover, the role of various culture media and serum supplement is essential for optimized growth and differentiation of primary organotypic cultures [96].

Recent and rapid advances in culture technology have permitted the generation of human skin equivalents in vitro, consisting of collagen gels with incorporated fibroblasts covered by proliferating and differentiating keratinocytes [97]. Rebuilding of graft material is one of the key elements of successful outcome of this new technology [94]. The aim is to create a tissue-engineered substitute, which more closely resembles the normal regional microanatomy and physiology of the skin, allowing better integration to the host with minimal or no scarring [75]. Organotypic skin cultures result in fabrication of human epidermal tissues that mimic the biochemical and morphologic properties of human skin demonstrating clearly manifest epithelial–mesenchymal interactions and offering new possibilities for wound treatment [94,98]. In that regard, novel therapeutic manipulations are being investigated to improve the degree of integration between a tissue engineered dermal construct and the host by both molecular manipulation of growth factors but also by understanding and harnessing mechanisms of regenerative biology [75].

In search of the best scaffold for organotypic skin culture, a novel composite xenogenic collagen-based material with unique properties has been created and used to reconstitute full thickness human skin in vitro [94]. Based on long established technology used for the production of collagen dressings for the treatment of burns, this novel, composite material offers excellent growth support of highly biodegradable spongy layer, combined with mechanical strength of collagen membrane. The use of the substrate enables to obtain organotypic culture that resembles full thickness skin with fibroblasts layer and well-developed multi-layer epithelium [94].

It is obvious that replacement of both epidermal and dermal layers is important for achieving optimal take of cultured grafts and for optimizing the quality of wound healing. Development of complete dermal–epidermal skin replacement (composite grafts) will undoubtedly greatly simplify burn management. Skin equivalent (organotypic) cultures generate human skin grafts that shortly after grafting normalize their tissue architecture, basement membrane structure and barrier function [99]. Clinical application of this modality has already been tested. An organotypic skin substitute consisting of allogeneic dermal fibroblasts embedded in a collagen gel overlain with allogeneic epidermal keratinocytes has been used to cover successfully clean elective wounds (tattoo removal) with documented allogeneic cell survival up to 2.5 years after grafting [100]. Limited application of composite cultured skin (CCS) allografts to correct childhood hand syndactyly and flexion contractures characteristic of dystrophic epidermolysis bullosa, has been also reported with good to excellent results [101]. A short series using the same allogenic composite model applied to burns patients with less than 20% total body surface area affected has been recently reported, however, with little effective take. It is obvious that further development of this model is

needed to overcome the hostile wound bed seen in burns patients [100].

The major limitation for the application of an autologous in vitro tissue-engineered reconstructed skin (RS) for the treatment of burnt patients is the delayed vascularization of its relatively thick dermal avascular component, which may lead to graft necrosis [102]. A new human endothelialized reconstructed skin (ERS), combining keratinocytes, fibroblasts and endothelial cells (EC) in a collagen sponge has been recently developed, resulting in early vascularization of the ERS most probably the result of inosculation of the capillary-like structures CLS network with the host's capillaries, rather than neovascularization, which is a slower process. These results open exciting possibilities for the clinical application of many other tissue-engineered organs requiring a rapid vascularization [102].

8. Dermal regeneration templates (artificial skin substitutes)

The recovery of skin function is the goal of each burn surgeon. Split-skin graft treatment of full-thickness skin defects, a standard treatment method still widely applied, leads, however, to scar formation which is often vulnerable and unstable [103]. It frequently leads to severe debilitating contractures due to lack of adequate dermal support. The clinical use of artificial skin substitutes has been celebrated enthusiastically as an improvement in burn therapy over the last two decades [104]. It has been successfully used to permanently replace skin destroyed by burn ranging from 10 to over 95% TBSA [105]. Artificial skin is a bilaminar membrane made of dermal and epidermal portions. An example of the dermal portion is a porous fibrous matrix arranged in a three-dimensional pattern closely resembling the fiber pattern of normal dermis. A thin silastic covering serves as a temporary epidermis [103,105–107]. When grafted on an excised wound, it becomes with time populated by the patient's own fibroblasts [105]. An autogenous ''neodermis'' is thus produced as fibroblasts and vessels migrate from the wound bed into the artificial dermal template. Using the artificial fibers as a scaffolding, migrating autologous fibroblasts synthesize new connective tissue in the collagen fiber pattern of normal dermis rather than the pattern of scar, while slowly biodegrading the artificial fibers. This replacement dermis functions as normal dermis and not as scar tissue [105]. In practice, the rate of dermal tissue formation and scarring is influenced directly by the rate of scaffold angiogenesis, degradation, and host response induced by the scaffold materials [103]. The patient's own epidermal cells, subsequently seeded or grafted on the ''neodermis'', grow into a confluent epidermal layer producing a permanent skin replacement characterized by an anatomically functioning dermis and epidermis [105].

There are various biopolymer dermal regeneration templates (artificial skin substitutes) commercially available [103,108] readily providing biocompatible material in unlimited quantity that can be tailored to the particular wound site [107]. These artificial skin substitutes are effective for management of contractures, chronic wounds and chronic skin illnesses. They decrease or avoid the risk of donor area morbidity which is more difficult to treat in children such as a deep donor site wound with attendant potential for infection, scarring and permanent pigment changes; provide long-term coverage of the wound; and can be used in conjunction with autologous tissue [107,108].

Integra (Johnson and Johnson, Hamburg, Germany; Integra Life Sciences Corporation, NJ, USA) is a biopolymer tissue-engineered bilayer (bilaminate) material consisting of a collagen and chondroitin-6-sulfate dermal regeneration template with a temporary silicone epidermal layer [103,106,107]. After neovascularization of the dermal component within 28 days in average, the silicone layer is removed and replaced with an epidermal autograft to reconstitute permanently the epidermal coverage [107,109,110]. Acellular dermal replacements perform well with extremely thin autologous split-skin grafts. The results, however, have been inconsistent when combined with cultured epithelial autografts [81,83]. No controlled trials have yet demonstrated any long-term benefits for the use of Integra. Reports are merely anecdotal showing that it could be a valuable alternative in certain situations particularly for secondary reconstruction and treatment of burn scar contractures. Some clinical trials report even average results [111]. In a clinical report about 12 consecutive facial burns the texture of the resultant scar was found to be good but not as supple as thick autograft. It was also concluded that Integra was not well suited for use in the coverage of eyelid burns [112]. Its use dramatically increases costs, both directly for the product and indirectly for the increased hospital time and operations. Additional disadvantages of Integra are the necessity of two operations, risks of infection, and time-consuming dressing changes [113].

One month postoperatively, naturally-formed collagen fibres can be observed in the dermal regeneration template. By one year, host collagen typically replaces the Integra matrix completely, and elastic fibres become evident throughout the neodermis [107]. Integra is indicated as an immediate and temporary coverage for acute wounds [109] as well as for extensive full thickness burned patients and for post-traumatic reconstruction and contracture release procedures [81,87]. Obviously, the successful use of this modality rests on the take rate as well as on the rate of infection. Fibrin glue and negative-pressure therapy has been shown to improve the take rate and time to vascularization even in complicated wounds [106,114] following which autologous epidermis could be applied. An ingeneous novel micrografting technique taking advantage of this technology has been described for the treatment of head and neck full-thickness burn injury. The burn wound is reconstructed with the dermal template followed by early implantation of microdissected hair follicles through the

silicone epidermis resulting in complete re-epithelialization and a hair-bearing scalp without the need for a split-thickness skin graft [115]. In general, coverage of full-thickness wounds having a well-vascularized surgical bed with Integra results in functional and aesthetic outcome with good skin quality [116]. Thick autograft remains, however, the gold standard in management of deep facial burns. Integra, on the other hand, may provide an acceptable alternative resulting in excellent color and minimally visible skin graft junctures excluding eyelid burns. Integra usually confers functional and cosmetic benefits similar to those of full-thickness grafts without comparable potential for donor-site morbidity [110]. Even though, the texture of healed full thickness wounds with Integra is good, it is not, however, as supple as those covered by thick autograft [112]. Integra requires also extended post-operative care to prevent recontraction [117], nevertheless, it is an important reconstructive dermal substitute for the severely burned or post-traumatic patient if handled by a skilled surgeon in a correct way [104].

Other artificial skin substitutes include Alloderm (Life Cell Corporation, Woodlands, TX) (cryopreserved acellular cadaveric dermal matrix). It serves as a scaffold for the ingrowth of cells and blood vessels and is usually combined with autografting. Dermagraft (Advanced Tissue Sciences, La Jolla, CA) (neonatal foreskin fibroblasts cultured on a polyglactin mesh) is usually combined with meshed autograft. Terumo (Terumo, Tokyo, Japan) (bovine collagen analog) and Pelnac (Kowa Company, Tokyo, Japan) (silicone epidermis and collagen matrix dermis) are also combined with thin epidermal autografts to close difficult wounds and ulcers [20]. Artificial skin appears to provide a successful physiologic and cosmetic skin replacement in severe burn injury [105]. Application of these products in burn treatment, however, is still limited to specific indications though results are rather encouraging. A major limiting factor for their widespread utilization is undoubtedly their high cost.

9. Bilaminar skin substitutes—dressings

The simple objective of these products is to replicate the function of skin as closely as possible while healing takes place. These products are easy to use, readily available, and technologically simple. They are theoretically valuable in the management of second degree burns, however, may be responsible partly for some morbidity [20]. Biobrane (Dow Hickam, Sugarland, TX) (outer silicone film and inner layer of nylon and collagen) has a shelf life of three years. Transcyte (Smith & Nephew, Cargo, FL) (similar to Biobrane with added biologic layer derived from neonatal fibroblasts) is stored at −80 °C. Laserskin (FIDIS Advanced Biopolymer, Abano Terme, Italy) (sheet of benzyl esterified hyaluronic acid perforated by laser then seeded with non-proliferating fibroblasts) can be used as an engineered bilayered graft or as a matrix for application of cultured autologous keratinocytes [20]. Cultured cellular sheets composed of mixture of autologous or allogenic keratinocytes and fibroblasts seeded on a polyurethane membrane (mixed culture sheet) have been useful for the treatment of split-thickness skin graft donor wounds to accelerate the epithelization process. This may be extremely useful in extensive burn injuries where available donor skin is so limited such that skin grafts need to be harvested repeatedly from the same area of unburned skin [16].

10. Cytokine and gene therapy

Major thermal injury is a particularly severe form of trauma characterized by high cardiac output, increased oxygen consumption, and protein and fat wasting. This vulnerable hypermetabolic state compromises the immune system and attenuates wound healing. Moreover, it causes tissue damage by membrane destabilization and energy depletion at the cellular level, resulting in tissue necrosis [118,119]. A logical therapeutic approach to promote recovery after burn trauma would therefore, be to block the immediate triggering of the inflammatory cascades that result in prolonged metabolic imbalances. A second component of the therapy would be to enhance wound healing, several molecular elements of which are regulated in part by components of the inflammatory cascade [118].

As our knowledge of the basic mechanisms of wound healing and body's response to injury is expanding to the bio-molecular level, new prospects for therapy are emerging. It is not science fiction any more to imagine that the effect of ''positive'' growth hormones and cytokines may be enhanced and that of ''negative'' factors suppressed through molecular or genetic manipulation [4]. Data suggest, for example, that the presence of the epidermal growth factor (EGF) receptor is a common denominator in the wound healing process after burn injury. When coupled with the clinical evidence of accelerated re-epithelialization following exogenous application of EGF, findings suggest an endogenous growth factor mediated pathway during wound repair that may be amenable to exogenous manipulation [120]. As promising as it may be, research into this domain has yet to overcome numerous obstacles the least of which is still our incomplete understanding of the intricate mechanisms involved.

Local application of cytokines as proteins (transforming growth factor-TGFβ, heparin binding epidermal growth factor-like growth factor-HB-EGF, etc.) has been shown to be ineffective and of little clinical value due to enzymes and proteases locally present in the wound and because of lack of adequate receptors [118,121,122]. Large amounts of systemic IGF-I needed for the desired therapeutic effects result, however, in serious side-effects, such as hypoglycemia, mental status changes, edema, fatigue and

headache. These adverse side-effects limit the therapeutic utility of IGF-I in the treatment of burns [118].

Gene therapy is emerging as an effective therapeutic approach to improve clinical outcomes after thermal injury [123]. Particle-mediated gene transfer in burnt skin is feasible and may provide a means of introducing biologic agents into injured tissue capable of enhancing bacterial clearance and improving wound healing [124]. Gene therapy to the skin, or to any other organ, is dependent, however, on a number of factors. All gene delivery systems have the following aims. Firstly, they should be able to accept a suitable therapeutic gene and not be restricted by the size of the gene. Secondly, therapeutic genes need to be expressed at the correct level for the right amount of time. Finally, the therapeutic gene must be in a delivery vehicle that is taken up by cells [125].

Gene transfection is a promising therapeutic approach. There are, however, several obstacles to overcome before this approach can be effective. Major obstacles are the selection of an appropriate vector for gene delivery as well as an appropriate delivery mechanism. Viruses have been used as delivery vectors [118,123]. Viral infection associated toxicity, immunologic compromise, and possible mutagenic or carcinogenic effects, however, make this approach potentially dangerous [126]. The use of naked non-encapsulated DNA or plasmid DNA constructs alone, without viral genes, have been used topically or delivered with a pneumatic 'gene gun'. Both have proven to be inefficient, perhaps due to the fragility of the naked DNA constructs in the extracellular environment and the traumatic consequences of 'gene gun' discharges on cellular integrity. Non-viral liposomal cDNA genes are stable complexes [118,122]. Their use, however, has been limited due to their low in vivo transfection efficiencies [118]. Modification of the standard liposomal structure to a cationic structure and the inclusion of cholesterol, together with the use of cytomegalovirus promoters in the cDNA constructs used for gene transfer, have nevertheless increased the efficacy and transgenic expression levels [118,127].

Bombardment by gene gun at various helium pressures (200–600 psi) is one route into the skin [124,128]. Other direct physical ''injection'' techniques include subcutaneous injection at the burn wound margin, the use of microseeding, microfabricated needles, and puncture-mediated DNA transfer [126,128–131]. Transport of macromolecules, as yet untested with DNA, has been achieved using depth-targeted pulsed electric fields and ultrasound. The development of gene-activated matrices (biodegradable polymers incorporating a therapeutic gene) takes the possibilities for wound treatment beyond gene therapy and into the realms of tissue engineering [128]. Nucleic acid vaccine [132] is yet another promising modality to promote wound healing following burn. As we enter the new millennium, gene therapy will play a major role in the treatment of diseases and their sequelae, wherever topical delivery of DNA is feasible and whenever development of therapeutic gene cassettes and delivery vehicles is economically viable [128].

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