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PROSPECTS

Epidermis Generated In Vitro: Practical Considerations and Applications

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Abstract The technology for culture of epidermis is one of the most advanced to date for generation of a tissue in vitro. Cultured epidermis is already used for a number of applications ranging from use as a permanent skin replacement to use as an organotypic culture model for toxicity testing and basic research. While simple epidermal sheets have been grafted successfully, more advanced models for skin replacement consisting of both dermal and epidermal components are in development and being tested in a number of laboratories. One of the most advanced in vitro models is the living skin equivalent, an organotypic model consisting of a collagen lattice contracted and nourished by dermal fibroblasts overlaid with a fully formed epidermis.

Key words: epidermis, skin, skin graft, cell culture, in vitro

The integument, the largest organ in the body, has long been the subject of biological and medical interest as a tissue for transplantation, reconstruction, and pharmacologic manipulation, and as a tool to assess properties of environmental toxins, cosmetic, and other formulations. The epidermis is a complex tissue that develops a stratum corneum at the interface of the skin and atmosphere, affording the primary barrier to the environment. The principal cell type of the epidermis is the keratinocyte, which makes up the stratified epidermis and the stratum corneum.

The keratinocyte has been the subject of considerable basic research because it undergoes many of the normal processes of differentiation in vitro. Keratinocytes enlarge as they differentiate, much as they do in vivo. They also terminally differentiate and form cornified envelopes [1].

Terminal differentiation of the keratinocyte in vitro requires calcium. Calcium allows stratification by assisting the formation of desmosomal contacts [2] and promotes differentiation [3]. For example, it specifically causes the induction of type I transglutaminase (the enzyme responsible for cornified envelope formation) and is necessary for the enzyme's activity [4].

Physical conditions can also influence the level of development achieved in vitro. Epidermal cultures cultivated at an air-liquid interface show enhanced organization and differentiation of the stratified layers [5]. The proper lipid profile of the epidermis including the presence of acylglucosylceramides and acylceramides, important in the formation of a barrier competent stratum corneum, is also achieved by culture at the air-liquid interface [6].

It has been postulated that one reason for enhanced epidermal development at the air-liquid interface is the establishment of chemical gradients in the epidermis [7,8]. Evidence in vivo indicates that a calcium gradient exists as a result of water loss in the more superficial layers [9]. Also, evidence in vitro has shown that while low concentrations of calcium can inhibit differentiation and high concentrations can lead to terminal differentiation, exposure to intermediate levels may be beneficial in inducing biochemical maturation [7]. The rate of transepidermal water loss can also influence lipid biosynthesis [10]. Retinoids are potent inhibitors of epidermal differentiation, and it has been proposed that the lack of contact of the superficial layers with retinoid-containing medium in cultures raised to an air interface may promote differentiation [8].

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Recent interest in the generation of skin in vitro for transplantation has been sparked by the pioneering work of Green et al. [11], Bell et al. [12], Hefton et al. [13], Thivolet et al. [14], and others. Patients with extensive burns require graft material other than autologous skin. Allograft skin from cadavers and redundant skin from living donors have been used as temporary skin replacements. However, the development of a permanent skin replacement is important to the long-term survival of severely burned individuals. Ideally, we wish to develop a skin graft that would be permanent, readily available, and immunologically neutral.

KERATINOCYTE CULTIVATION

Advances in the development of keratinocyte cell culture have provided the technology for permanent skin replacements using cultured keratinocytes. For this purpose, the rapid cultivation of keratinocytes in large quantities becomes essential. This capability is dependent on the ability to maintain an adequate proportion of small, proliferative keratinocytes during subcultivation. The 3T3 feeder cell method [11] has been widely used for generation of epithelium for this purpose. The disadvantage of this method is that it requires serum and employs a xenogeneic cell type that may contribute antigenic components to the epithelium. The other most widely used method is the serum-free method of Boyce and Ham [3,15]. This method, however, does not support the proliferative capacity achieved with the 3T3 method and has difficulty maintaining a population of small, proliferative cells at confluence in the presence of high calcium [16], something desirable in the generation of graftable epidermis.

We have recently developed a method for keratinocyte cultivation that allows rapid culture expansion and maintenance of a high proportion of small, proliferative cells in the presence of high calcium (1.88 mM) in a defined, minimally supplemented basal medium (MSBM) [17]. While MSBM may be useful in the cultivation of epithelial tissue for autografting, our effort has focused on the development of allogeneic tissue. The practicality and desirability of using allogeneic cells lies in the ability to store a large number of cells that can provide a reliable and consistent source of graftable cells. Neonatal foreskins are an excellent primary source since they are readily obtained and have a high proliferative capacity. The method of choice must allow for the expansion of donor tissue to the extent that a single source will provide enough tissue to render cost effective the screening of the cell banks for pathogens and tumorigenicity required before clinical use. In addition, the cells that are produced must remain proliferative and retain the ability to generate a differentiated epidermis. Use of a defined medium also is desirable.

The MSBM method meets these requirements as a culture medium for normal human keratinocytes. It permits the cultivation of large numbers of cells rapidly under defined conditions. These cells can subsequently be induced to develop a mature epidermis that maintains a proliferative basal cell population. The MSBM method can be used to routinely generate greater than 10 square meters of graftable tissue from a single human neonatal foreskin in 4–5 weeks. Three passages in culture provide approximately 10^{10} cells for cryostorage as a cell bank. Subsequent culture of these cells for one passage results in an additional tenfold increase in cell number, which is then sufficient to produce approximately 100–200 m^2 of living skin equivalent from a single source. This accentuates the practical advantages of generating allogeneic grafts. Creating stocks of frozen cells allows for a continuous supply of consistent, graftable tissue.

GRAFTED EPIDERMIS

Optimally, skin grafts made from cultured cells should satisfy performance criteria similar to those for living skin autografts. The graft must adhere firmly to the wound bed and be of significant substance to prevent wound contracture. The cultured epidermis should be multilayered and sufficiently differentiated to provide a protective barrier against heat and water loss as well as topical infection. The permanence of the graft depends on rapid vascularization and integration of the tissue, maintenance of viability and functionality, and the absence of immune rejection of graft material.

Cultured grafts have employed autologous as well as allogeneic epidermal cells applied either directly onto the wound bed or onto allogeneic nonviable dermis. Cultured epidermal sheets have been used to cover extensive burns and leg ulcers [18]. Faure et al. [19] reported that there was no ultrastructural or biochemical difference if autologous or allogeneic cells were used to epidermalize a wound.

The cultured epidermal sheets prior to grafting are multilayered but poorly organized and relatively undifferentiated [19,20]. A granular layer and stratum corneum is seen 5–7 days after grafting, and the biochemical organization improves during the next 2 weeks [19,20].

Compton et al. have extensively studied the long-term fate of autogenous epidermal sheet grafts [20]. Focal patches of basal lamina are present 14 days after transplantation. The basal lamina is continuous by 4 weeks. Mature anchoring filaments, however, are not present until 1 year post-grafting.

Bilayered skin replacements, consisting of both an epidermal and a dermal component, have also been used. This approach has been explored using composite frozen allogeneic dermis with autologous epidermis [21,22] or allogeneic reconstructed dermis overlaid by allogeneic epidermis [12].

In composite grafts of autologous epidermis on allogeneic cadaver dermis, cadaver skin was grafted onto the wound bed. The epidermis was later removed and replaced with sheets of cultured autologous keratinocytes [23]. The grafts were firmly adherent and developed a viable, mature epidermis with a granular layer and a stratum corneum. Cadaver dermis, however, is limited in its availability, reproducibility, and safety. It is also often used in a two-step process, requiring epidermalization at a later date. Therefore, a dermal substrate constructed in vitro for forming a full thickness (bilayered) graft is desirable.

Biosynthetic wound coverings consisting of collagen and chondroitin-6-sulphate were first developed as a permanent wound dressing to provide an improved substrate for subsequent autografts [18]. Hansborough et al. [24] have cultured keratinocytes on this matrix material. Grafts of this material in athymic mice developed a stratified, differentiated epidermis and a neo-dermis similar to unwounded dermis [24].

An organotypic living skin equivalent, consisting of a dermal equivalent containing dermal fibroblasts and type I collagen overlaid with epidermal cells, has been grafted onto wounds in animals [12,25] and humans [26,27]. After grafting, the epithelium is stratified and well developed [12]. Basal lamina is present by 7 days [28]. The dermal portion is quickly vascularized and may allow for earlier permanent coverage of the wound site. Although epithelial sheets may be used alone, epithelium applied with a dermal substrate provides the benefits of durability and more effective wound closure. It also may speed the healing process by providing the appropriate connective tissue cell, the dermal fibroblast, within a framework that would promote appropriate remodeling and wound repair and prevent wound contracture.

IMMUNOLOGICAL CONSIDERATIONS FOR GRAFTING

Allogeneic as well as autologous cell sources should be evaluated. Cultured human allogeneic epidermis has been used on second- and third-degree burn wounds [27] and leg ulcers [14]. Cultured mouse epidermal cells have been used to demonstrate successful grafting across major histocompatability barriers [29]. A composite of frozen allogeneic dermis and autologous epidermis has been tested in mouse as well as in humans on thermal burns [21,22]. Bilayered allogeneic grafts of living skin equivalent have been successfully grafted in rats [12] and in man [26]. These reports showed the successful development of a fully differentiated epidermis with a mature dermis when composite grafts were used.

The prolonged survival of allografted epidermis does not address the possibility of host reepithelialization. Hammond et al. [29] used a silicone transplant chamber in the mouse, physically separating the recipient epidermis from the graft, therefore ruling out the possibility of host cells overgrowing the graft area. In the grafting of human cells on patients, separation between recipient and donor epidermis is not feasible and progressive replacement of the grafted cells by host cells can occur over a period of months [30]. It has been postulated that in decubitus ulcers, for example, the presence of donor epithelium may promote wound healing by the host cells through the release of cytokines [31]. Cultured epidermis may provide, therefore, both physical and biological benefits to the wound site.

The successful grafting of cells across major histocompatibility barriers suggests that grafted cells are either nonimmunogenic or so weakly immunogenic that immunologic rejection could not be detected. In skin, initiation of the immune response has been primarily attributed to class II antigen-bearing Langerhans cells [32] and to subsets of immunogenic cells in the dermis. These include macrophages, lymphocytes, and capillary endothelial cells. The failure of cultured keratinocytes to be rejected could be associated with the loss of cells expressing class

II antigen during culture. In vitro culture has been shown to reduce the subsequent immunogenicity of grafts [14]; it has been demonstrated that cultured epidermal cells are devoid of Langerhans cells and other class II MHC-bearing cells [19,33]. Culture conditions aid in the removal of these cells, but it is not clear whether cultured cells also show a loss of antigens on their surface [34]. Hammond et al. [29] have shown that freshly isolated cells induce accelerated rejection of whole thickness skin grafts, whereas cultured cells fail to prime allogeneic mice. Thivolet et al. [14] have also shown that cultured epidermal cells, in contrast to freshly excised epidermis, fail to stimulate an allogeneic response of peripheral blood cells. The absence of stimulation was noted on the day of grafting as well as at later times.

Keratinocytes and dermal fibroblasts do not constituitively express class II antigens. When tested in vitro, these cells fail to trigger an allogeneic response even when class II antigen expression is induced by gamma interferon [33].

Both in vivo and in vitro data therefore suggest that parenchymal and stromal cells such as the keratinocyte and dermal fibroblast, may lack the antigenicity necessary to elicit an immune response. These cells would therefore be feasible for allograft use, providing all passenger cells are removed by cultivation.

FURTHER DEVELOPMENT OF THE LIVING SKIN EQUIVALENT MODEL

Previous attempts to use a collagen lattice to form a full-thickness living skin equivalent have often failed to generate a reproducible, uniform, well-developed epidermis on the lattice. In addition, regardless of what dermal material is used, preventing the migratory epidermis from growing around the underside of the dermal matrix is a necessity.

We have successfully developed a reproducible, large-scale method for the formation of fully formed living skin equivalents (LSE) as outlined in Figure 1 and described in detail elsewhere for in vitro testing (LSE-T) [35]. The LSE is formed in a suspended chamber on a porous polycarbonate membrane. Although the dermal equivalent contracts slightly away from the walls of the suspended chamber, the lattice remains sufficiently adherent to the membrane to keep it in place and inhibit the epidermis from growing around the underside of the lattice [36]. The membrane also supports the LSE when

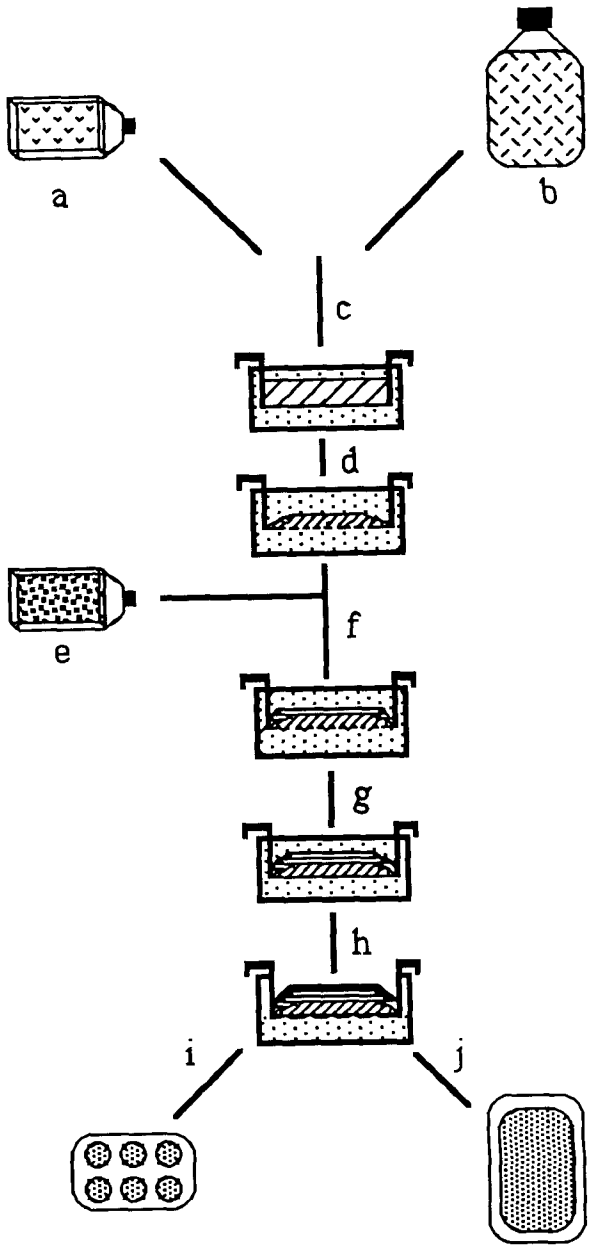

Fig. 1. Diagram of the formation of a living skin equivalent. **a:** Cultured dermal fibroblasts are derived from neonatal foreskin. **b:** A solution of purified acid-extracted bovine type I collagen. **c:** Fibroblasts and media are mixed with the collagen solution and cast into the suspended chamber where the neutralized mixture gels. **d:** The fibroblasts contract the collagen matrix to form a dermal equivalent in approximately 4–6 days. **e:** Keratinocytes, derived from neonatal foreskin are serially passaged in MSBM. **f:** Epidermalization: keratinocytes are seeded onto the surface of the dermal equivalent at 0.5–1.0 × 10^5 cells/cm^2 and cultured for 2 days in MSBM-0.08 mM Ca^{++} to promote even coverage of the large lattice surface. (LSE-T does not require a low-calcium step.) **g:** Stratification: cultures are incubated for 2 days in MSBM-1.88 mM Ca^{++} to allow stratification of the epidermis. **h:** Maturation: the LSE is cultured at the air-liquid interface to promote organization and maturation of the epidermis. **i:** LSE-T is fully mature after 10–14 days at the air-liquid interface. **j:** LSE-G is suitable for grafting after approximately 4–7 days.

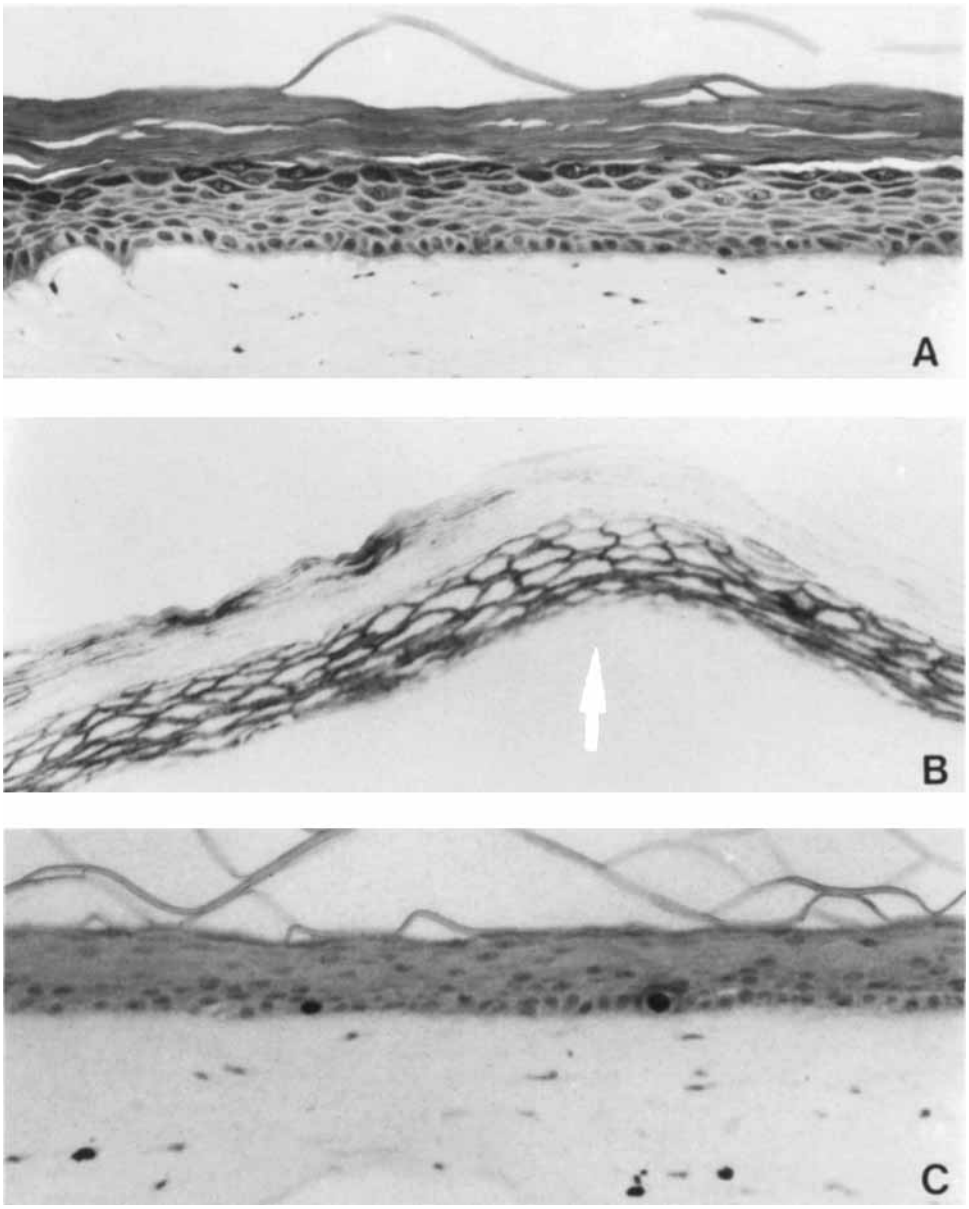

Fig. 2. Differentiation and mitotic activity of the living skin equivalent (LSE). **A:** Hematoxylin and eosin-stained paraffin section of the LSE showing a uniformly stratified epidermis containing a well-developed basal layer, stratum spinosum, stratum granulosum, and stratum corneum. Basal keratinocytes are firmly attached to the underlying fibroblast-containing dermal equivalent. **B:** Immunocytochemistry (avidin biotin horseradish peroxidase) on unfixed cryostat sections of an LSE labeled with monoclonal antibody to type I transglutaminase (BC.1) showing the normal differential expression of this primarily particulate enzyme in the suprabasal layers of the epidermis. LSE labeled with monoclonal antibody to type I transglutaminase (BC.1). Arrow indicates the epidermal-dermal junction. **C:** Autoradiograph of an LSE, demonstrating 3H-thymidine uptake (2 h incubation) by the basal keratinocytes and dermal fibroblasts after 16 days at the air-liquid interface. (All magnifications 200×.)

raised to the air-liquid interface during maturation. The development of the LSE for LSE-T requires that the skin equivalent be as mature and as fully differentiated as possible. While the LSE for graft (LSE-G) may not require the same degree of maturity, moderate differentiation is desirable and is easily achieved when the cultures are raised to the air-liquid interface (Fig. 2A,B). The organotypic cultures have a proliferation rate similar to that of normal skin with mitotic activity being restricted to the basal keratinocytes of the epidermal component and the dermal fibroblasts of the dermal component (Fig. 2C). As a result, a multilayered epidermis

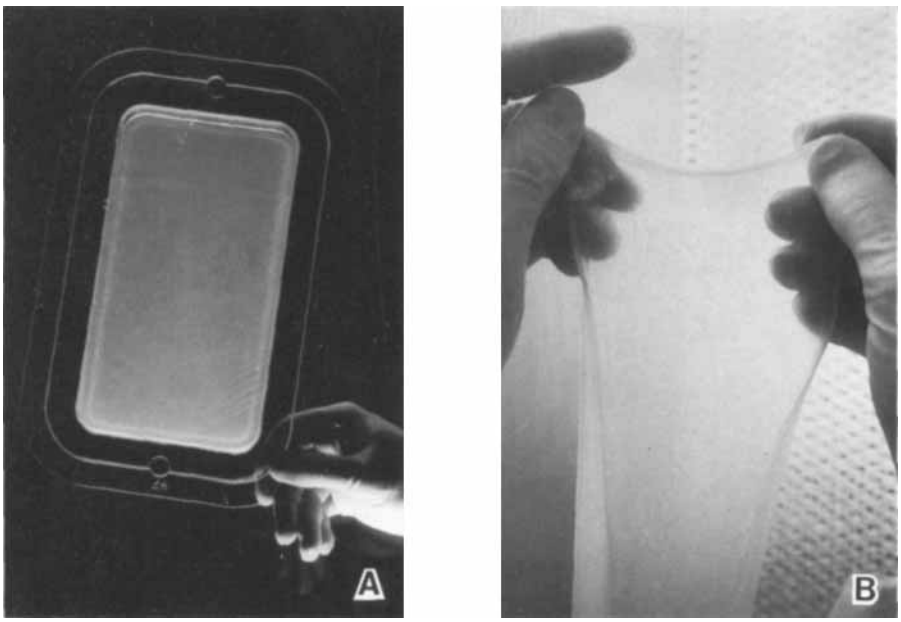

Fig. 3. LSE-G. **A:** The living skin equivalent in its custom designed form for grafting. **B:** Removed from chamber to show its consistency and handleability.

forms with proper morphological and biochemical organization.

LSE-G has been designed in a custom configuration that is a larger adaptation of the suspended chamber concept (Fig. 3A). This 4 × 8 inch (10 × 20 cm) sheet of bilayered tissue is grown, matured at the air-liquid interface, and transferred to the surgical site in the custom-designed suspended chamber configuration, providing asceptic protection and ease of handling. Procedures are conducted in a Class 100 environment to ensure sterility of the tissue and its container. The culture protocol is essentially the same as that used for LSE-T (Fig. 1). The LSE is allowed to mature at the air-liquid interface, by modifying the medium to support maturation and cornification. LSE-G is ready for use 14–16 days into the fabrication process and can be maintained in vitro for an additional 10–12 days.

The biological and physical properties of LSE-G are advantageous for clinical use: Overall thickness is 0.5–1.0 mm, making it similar to split-thickness skin grafts. The presence of a well-developed epidermis adds additional strength and durability to the tissue, protecting the proliferative basal cell population and providing a barrier to dessication and topical infection. Because of the consistency provided by the supporting dermal matrix, the tissue is easily removed from the supporting polycarbonate membrane and may be handled without damage to the epidermis (Fig. 3B). It also conforms exceptionally well to contoured body surfaces.

CONSIDERATIONS FOR THE FUTURE

Efforts are underway to define optimal conditions for cryopreservation of the dermal equivalent. Viable recovery rates as high as 85% have already been achieved. In addition for providing for long-term storage, successful cryopreservation would shorten the overall fabrication time of LSE-G, particularly if used in conjunction with cryopreserved epidermal cells. Incorporation of antibiotics within the dermal matrix would also provide additional therapeutic benefit to the patient as well as aid the stability of the dermal substrate by inhibiting production of bacterial collagenase in the wound bed.

Addition of pigment-producing cells (human melanocytes) to the living skin equivalent is being developed to provide advantages to both the in vitro and graft models. Provision of natural pigmentation would afford the grafted skin the necessary protection against ultraviolet irradiation.

Our current protocol employs small amounts of serum (0.1–2.0%) to maintain the viability of dermal fibroblasts and provide additional nutritional support during maturation. Although screened human serum may be substituted for the calf serum in graft material, a defined, serum-free protocol would be ultimately desirable.

Efforts are underway to modify MSBM to not only support the epidermal cells but also the fibroblasts in a defined environment.

Epidermis generated in vitro currently provides a host of biological and clinical opportunities. When used in conjunction with a dermal component, it holds great promise for further clinical development and expanded applications.

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