Keratinocyte-fibroblast Cocultures on A Bi-layered Gelatin Scaffold for Skin Equivalent Tissue Engineering

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Abstract

The skin is composed of three parts: epidermis, dermis and subcutaneous fatty layers. In order to develop a biomimetic material for more realistic tissue regeneration, we fabricated a bi-layered gelatin sponge with different pore sizes by freeze-drying at different temperatures –20 ℃ and –196 ℃, respectively. A rete ridges-like topographic microporous structure, which provided the paracrine crosstalk in epithelial-mesenchymal interactions similar to the basement membrane in epidermal-dermal junction, was formed between these two compartments. Chrondroitin-6-Sulphate (C-6-S) and hyaluronic acid (HA) were incorporated within the gelatin scaffold to create an appropriate microenvironment for cell proliferation and migration. The mechanical strength of gelatin sponges was improved by cross-linking of gelatin with 1-ethyl-3 (3-dimethyl aminopropryl) carbodiimide (EDC). The lattice structure and pore sizes were evaluated by SEM to confirm the interconnected porous structures retained. We chose a dynamic spinner flask seeding method for more evenly distribution followed by a culture system in the air-liquid interface and cultured for 21 days. Differentiation and phenotypic expression of keratinocytes were investigated by histological analysis. In this study, we found a multiple epidermis-like layers constructed by cultured keratinocytes. It is suggested that the bi-layered scaffolds have the potential to be used as skin equivalents for the application in burn patients. In the future, the in vivo animal model tests will be evaluated.

***Keywords*:** Sponge gelatin, Glycosaminoglycan, Keratinocyte, Dermal fibroblast, Skin equivalent

Introduction

Nowadays, the treatment for patients with full-thickness burns, which includes both dermal and epidermal tissue engraftment, is autografting. Although split-thickness autografts is often the first choice for permanent wound closure in burn patients, this conventional approach is limited by the availability of donor sites. To overcome this source limitation of autologous skin, Rheiwald and Green in 1975 have shown serial and large culture of keratinocytes possible [1,2,3]. Since then, Skin reconstruction has undergone extremely rapid and spectacular progress. Although this method has been a tremendous step forward in the treatment of large burns, cultured epidermal sheets do not provide satisfactory skin coverage due to subsequent wound contraction [4]. The lack of dermis when these grafts are used on full thickness wounds seems to be another major problem [5]. In the following studies, the culture methods used involve prolong cultivation time in vitro resulting in a high percentage of differentiated keratinocytes, which had loosed their continuous proliferative capacities in epidermal renewal process. Complicated procedures for graft production and transplantation are also labor-intensive work. The allograft provides another choice and is also frequently used as a temporary cover of extensive burn until donor sites or cultured autografts are available [6,7]. However, there are still drawbacks of the allograft such as host-versus-graft reaction and is ultimately rejected. An ideal human skin substitute should be non-antigenic, biocompatible, durable, adherent, readily available and inexpensive [8-10]. The living cells included in each reconstructed skin substitute must retain their morphology, proliferative capacities and expression of tissue-specific functions. Thus, the culture conditions including the media, additives, matrix and three-dimensional environment have to be properly assessed and selected [11]. In this study, we would like to investigate the possibility of fabricating a bi-layered gelatin scaffold seeding with dermal fibroblasts and epidermal keratinocytes as a model of skin equivalent. The objective of this study is to develop suitable biological substitutes to restore, maintain and improve skin tissue functions.

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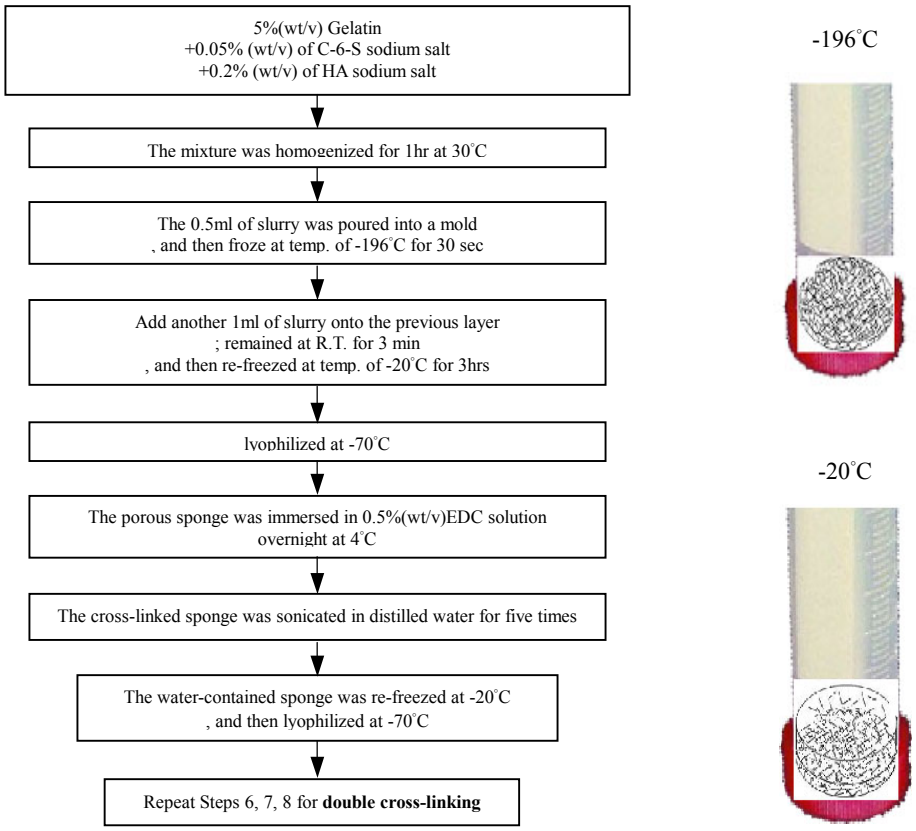
Table 1. The procedure of fabrication and cross-linking process of porous bi-layered gelatin-C6S-HA sponge



Materials and Methods

Fabrication and cross-linking of porous bi-layered gelatin-C6S-HA sponge (Table 1)

Gelatin, chondroitin 6 sulfate, hyaluronic acid, and 1-ethyl-3 (3-dimethyl aminopropryl) carbodiimide are all purchased from Sigma Chemical Co. (St. Louis, MO, USA). In brief, 5 wt% Gelatin (300 Bloom, from porcine skin) was dispersed in distilled water at 25 ℃. 0.05 wt% of C-6-S sodium salt (from Shark Cartilage) and 0.2 wt% of HA sodium salt (from Rooster Comb) were added to the dispersion. The mixture was homogenized using a Stirrer/Hot Plate for 1 hr at 30 ℃. The 0.5 ml of slurry was poured into an inverse 50 ml Centrifuge Tube (cap diameter 3 cm), and then froze at temperature of -196 ℃ for 30 sec. Added another 1 ml of slurry onto the previous frozen layer and remained at room temperature for 3 min. Froze the total volume of 1.5 ml scaffold at -20 ℃ for 3 hours followed by lyophilizing at -70 ℃. The fabricated porous membrane-like sponge was immersed in 0.5 wt% 1-Ethyl- (3-3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) solution overnight at 4 ℃. The cross-linked sponge was sonicated in distilled water for 3 min five times to remove the residual EDC. The water-contained sponge was re-froze at -20 ℃, and then lyophilized at -70 ℃. Repeat Steps for double cross-linking.

Culture of Keratinocytes and Fibroblasts (Figure 1)

Human adult foreskin biopsies were washed in multiple changes of phosphate buffered saline with penicillin-streptomycin-gentamicin antibiotics (Gibco Invitrogen Co.). Specimens were then chopped into 1- to 2-mm small pieces. The skin fragments were floated on 40U/mg thermolysin (Sigma) at 4 ℃ overnight. The epidermis was separated from the dermis with forceps and further incubated with 0.05% trypsin / 0.1% EDTA (Gibco Invitrogen Co.) for 15 min at 37 ℃ to dissociate keratinocytes. After centrifugation and trypsin inhibition, cells were plated for primary cell culture. Keratinocytes were cultivated by commercially available serum free/chemically defined culture medium with indicated medium supplement for expansion (EpiLife, Sigma). The culture medium is basically formulated on MCDB 153. On the other hand, the dermis was treated with 0.2% collagenase at 37 ℃ for 1 hr to obtain fibroblast cells. After centrifugation and enzyme inhibition, dermal fibroblasts were cultured routinely in DMEM supplemented with 10% FBS at 37 ℃, 5% CO2.

Skin equivalent fabrication

Dermal analog is modified from the bi-layered artificial skin developed by Burke and Yannas [12, 13]. In brief, fibroblasts were passaged and inoculated onto gelatin-C6S-HA substitute to form a dermal equivalent. After 3 days later, keartinocytes were then seeded onto the previous dermal equivalent and maintained under submerged conditions for 3-5 days at which point they were raised to the air-liquid interface for further cultivation until 21 days. The seeding method was a

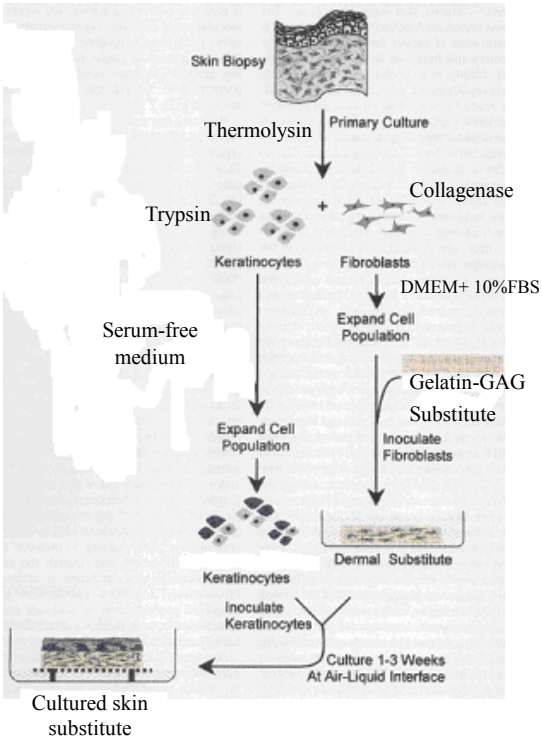
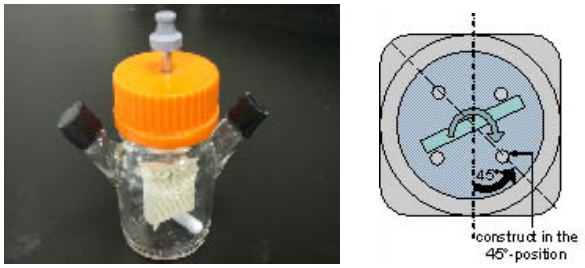


Figure 1. Cell culture of keratinocytes and fibroblasts



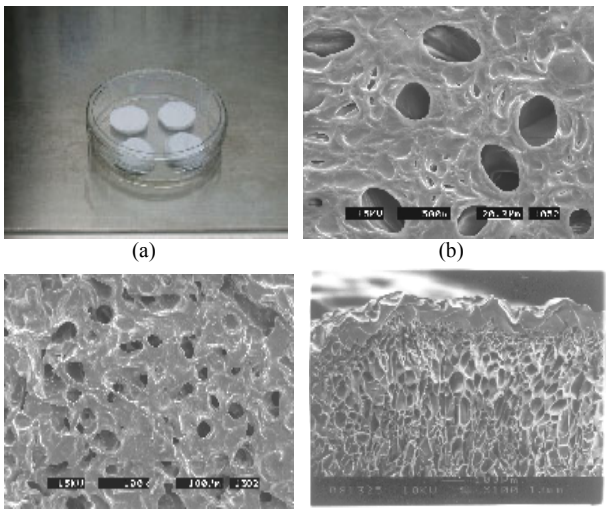
(a) (b)

Figure 2. Spinner flask bioreactor is cell-culture system designed to enable the seeding and the growth of engineered tissues. (a) Dynamic seeding method with spinner flask (b) Top view of the bioreactor; the construct is at 45^0-position for maximum fibroblasts adhesion density.

dynamic one in which scaffold was placed into 125 ml spinner flask, each with 80 ml of media, and cell density of fibroblasts are 2 x 104 cells/ml (Figure 2). The sample was stirred for 24 hrs at approximately 80 rpm under incubation conditions. The reconstructed skin substitute is obtained by seeding keratinocytes (106 cells/cm2) on the top of the fibroblasts-populated sponge and culture at the air-liquid interface for epidermaliztion [14].

Histology and scanning electron microscope (SEM) observation

The morphologies of the gelatin-C6S-HA sponges were characterized by a scanning electron microscope. The SEM samples were fixed in 4% formaldehyde for 1 h and then dried with a serious of increasing graded alcohols followed by



(a) (b)

(c) (d)

Figure 3. (a) The stereo appearance of fabricated gelatin-C6S-HA sponge. Scanning electron micrograph of gelatin-C6S-HA sponge freeze dried at –196 ℃ (b) and –20 ℃ (c), respectively. Rete ridges-like structure was indicated by the *arrow* shown in cross sectional view (d).

critical-point drier processing. The surfaces of sponges were coated with ultrathin layer of gold/Pt in an ion sputter. The average diameter of pore sizes was analyzed with arbitrary matrix zone. The histology specimens were treated in 10% formalin at 25 ℃1 hour. They were then embedded in paraffin and 3~5 μm sections were taken across or parallel to the specimen. The sections were mounted on slides and stained with hematoxylin and eosin.

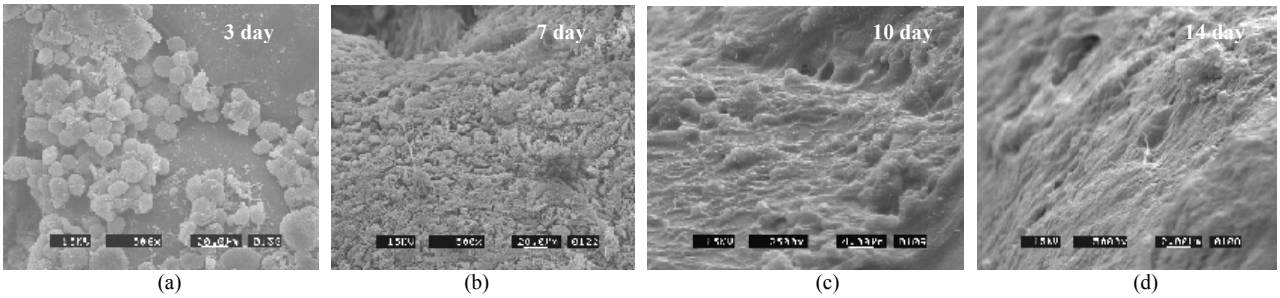
Results

Morphology of the gelatin-C6S-HA matrices

The gelatin-C6S-HA matrices retaining porous structure were successfully fabricated by this method. The fabricated sponges were shown below (Figure 3a). The porous gelatin-based sponge was approximately 1~2 mm thick. The morphology of lyophilized bi-layered gelatin scaffolds was investigated by measuring the porosity and porous structures of the matrix (Figure 3b,c). SEM showed that matrices had interconnected pores with mean diameters of 20μm, 150μm, respectively, depending on the different freezing temperature. The papillary-like structures similar to the native skin basal lamina controlled the gross surface features of the skin equivalent and the connected rete ridges-like structure was found (Figure.3d). It is also believed that infolds topography could enhance the differentiation and stratification of epithelial keratinocytes [15].

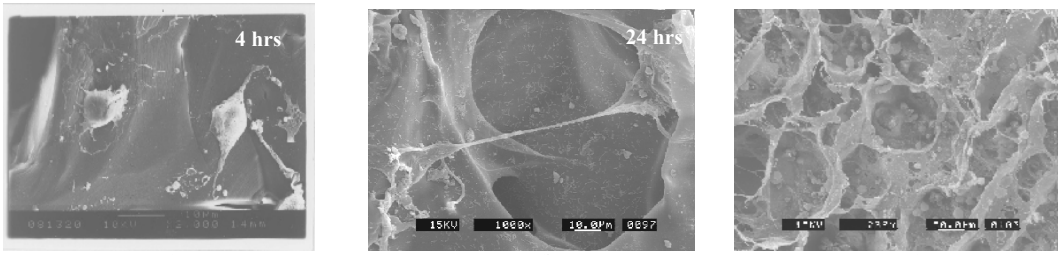
Proliferation and differentiation of Keratinocytes

Human keratinocytes seeded onto gelatin-C6S-HA lattices were able to attach to our biomaterial, grow, and expand in number (Figure 4). Expansion occurred within a mean time of 3 days to a subconfluent monolayer and became confluent after 7 days. The morphological characteristics of the differentiating suprabasal layers and the cornified



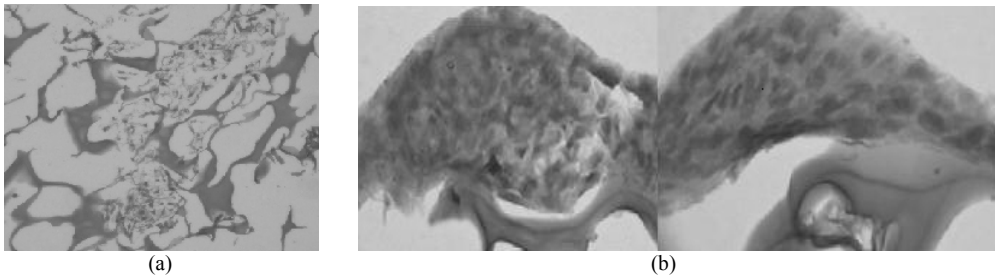
(a) (b) (c) (d)

Figure 4. (a) Basal keratinocytes attached to gelatin-C6S-HA matrices and secreted their own ECM proteins to build up basement membrane. (b) Proliferation to confluence on day 7th. Suprabasal keratinocytes differentiated to multiple stratified layers was observed on day 10th (c) and day 14th (d).



(a) (b) (c)

Figure 5. (a) (b) Dermal fibroblasts stretched out their filopodia and filaments during cell adhesion and migration process. (c) Evenly distribution of fibroblast cells was optimized by spinner flask seeding method



(a) (b)

Figure 6. (a) Human dermal fibroblasts proliferated and secreted their own extracellular matrix. (100 x) (b) Keratinocytes proliferated and differentiated into stratified epithelial layers. (400 x) (H/E stain).

squamous layer of the epidermis were found in the following 10 and 14 days. This suggested that normal keratinocyte maturation process was undergoing.

Migration and division of Fibroblasts

Initially, dermal fibroblasts started to spread out the filopodia immediately after attachment to the gelatin-C6S-HA scaffold within 4 hrs (Figure 5a). Stretching out of the anchoring structures during cell migration from one place to another had also been seen in 24 hrs (Figure 5b). This is believed to be beneficial to cell integrity and cell viability. The evenly distribution of fibroblasts throughout the porous gelatin-C6S-HA scaffold was achieved by the dynamic spinner flask seeding method (Figure 5c).

Histological analysis of skin equivalent

The bi-layered gelatin scaffold served to induce regeneration of the dermis and the epidermis. Histological examination shows the epidermal layer with stratification and dermal substrate containing gelatin plus cells. Usually 3 days, the gelatin-C6S-HA was biologically modified into a new neodermis by the fibroblasts. Dermal fibroblasts produced their own extracellular matrix and showed the rich cellular growth (Figure 6a). Basal keratinocytes proliferated and differentiated into stratified epithelial layers (Figure 6b). The stratified epidermal layer was composed of approximately 4 to 5 keratinocytes layers. The neo-epidermis increased in thickness within 3 weeks.

Discussion

Incorporation of C6S and HA

Cell adhesion is generally mediated by certain extracellular matrix proteins such as fibronectin, vitronectin, and laminin as well as various glycosaminoglycans[16]. In order to improve the biocompatibility, C-6-S is incorporated into gelatin to offer the opportunity to exploit the many biocharacteristics of this polysaccharide and create an appropriate environment for cellular signaling. Immobilized GAG also retains large porous lamellar matrix spaces, probably due to its water-binding capacity, which promotes matrix swelling. Hyaluronic acid, repeating disaccharide sequence, is endogenous to normal skin and has been

implicated in scarless fetal healing [17,18]. Fibroblasts appear to carry receptors, such as CD44, allowing specific attachment to and migration along HA molecules. Furthermore, CD44 ligands like HA and C-6-S may inhibit macrophage multinucleation as for reducing the inflammatory response [19].

Characteristics of gelatin-C6S-HA scaffold

In the process of harvesting cultured epithelial sheet grafts, the basement membrane proteins of the cultured graft may be inevitably digested by the digest enzyme thus affecting its anchorage on the recipient wound bed [20]. Shrinkage of the harvested cultured sheet and blister formation are two drawbacks of conventional cultured epidermal autografts. To avoid this potential negative aspect of enzyme treatment, we investigated the cultivation of keratinocytes directly on a membrane-like gelatin sponge. We also took the advantage of inoculating autologous dermal fibroblasts instead of using irradiated or mitomycin C-treated 3T3 cells as feeder layer. This procedure not only shortened the *in vitro* cultivation time but also limited the risk of hepatitis HIV transmission and other skin disease [21,22]. Dermal analog pre-seeded in vitro with proliferating autologous keratinocytes could allow one-stage engraftment, too. The diffusion of nutrition supply from the recipient wound is critical especially in the first few days when vascularization in the neodermis has not developed [23] The micropores invagination topography between dermal-epidermal layers showed the possibility of dynamic interactions in tissue homeostasis and served as papillary-like structures just as in normal dermal papillae, which resembled as rete bridges in the dermal-epidermal junction. The micropores (10~40 μm) allow the migration of the keratinocyte populations from the upper surface to the underneath recipient wound bed in the future clinical application. There are also some macropores (>120μm) for the drainage of wound exudates. Besides acting as feeder layer, the dermal fibroblasts of the gelatin sponge demonstrate the following functions: First, fibroblasts may secrete proteins such as fibronectin, laminin to enhance the attachment of cultured keratinocytes onto the neodermis. Second, they release cytokines such as beta-transforming growth factors, keratinocyte growth factors to modulate epidermalization [24,25]. Last but not least, the preparation time of bi-layered skin equivalent is within 21 days sooner than that of conventional cultured epithelial autograft, because the keratinocytes of gelatin skin equivalent are able to be grafted at their sub-confluent stage.

Future study

The main limitation of grafting a thicker reconstructed skin on wounds could be a delay in revascularization [26]. Therefore, the addition of endothelial cells to this model lead to the formation of capillary-like structure in the dermis and improve the take rate of skin substitutes would be considered in the future. Further work in the reconstructed epidermis will need to focus on the incorporation of melanocytes and Langerhans cells to study not only the complex mechanisms of epidermal cell-cell interactions but also the role of each cell type in the skin pigmentation and immune response [27].

Conclusion

Cultured human keratinocytes can be combined with collagen-GAGs in vitro and exposed to the air to stimulate epithelial stratification and cornification. [28-31]. Exposure of normal human keratinocyte cultures at the air-liquid interface induces terminal differentiation and results in a multilayer stratified tissue. This study demonstrates that autologous keratinocytes can be combined with well- characterized gelatin-G6S-HA matrices to result in a tissue with properties that in many ways simulate normal skin. It also suggests clinical applications *in vivo* for the treatment of extensively burned patients in the future. In general, this culture model has proved a very suitable tool for the characterization of fundamental events in epithelial- mesenchymal interaction controlling epidermal tissue reorganization and differentiation as well as tissue homeostasis.

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