Reconstructed Skin from Cultured Human Keratinocytes and Fibroblasts on a Collagen-Glycosaminoglycan Biopolymer Substrate

Steven Boyce a, Serge Michel b, Uwe Reichert b, Braham Shroot b, Rainer Schmidt b

aDepartment of Surgery, University of Cincinnati Medical Center, Ohio, USA; bCentre International de Recherches Dermatologiques Galderma, Valbonne, France

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Abstract. A lattice prepared from biopolymer substrate bovine skin collagen and chondroitin-6-sulfate (glycosaminoglycan) served as a support for normal human keratinocytes and fibroblasts. Air exposure of the lattice on an agarose block gave rise to reconstructed epidermis, the histological features of which are very similar to normal human epidermis. Indirect immunofluorescence staining of the plasma membrane-associated transglutaminase, the enzyme responsible for the synthesis of the cornified envelope, revealed the same tissue distribution as observed in vivo. Cell cycle analysis showed a large shift of the normal human keratinocyte population into S-phase and cell division during the 1st week post-inoculation. Furthermore, the effects of two modulators of differentiation (25-hydroxycholesterol and sodium butyrate) on the reconstruction of the epidermis were evaluated.

Introduction

Epidermis performs the most fundamental function of skin, as a barrier for control of vapor transmission and protection against the environment. Unique characteristics of human epidermis limit to a great extent the use of animal models for evaluation of new drugs, and safety testing of consumer products and cosmetics. These limitations have stimulated development of several in vitro models of human epidermis, i.e. (1) pure cultures of human epidermal keratinocytes (NHK) [1–3], (2) NHK or skin explants cultured on collagen gels contracted by human fibroblasts (NHF) [4–7], (3) NHK cultured on the epidermized dermis [8] and (4) NHK on collagen glycosaminoglycan (GAG) populated with NHF [9, 10].

To utilize cultured skin substitutes for applications including pharmacologic evaluation of drugs, safety testing of consumer products and cosmetics, and closure of skin wounds that require grafting, certain re-
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Requirements must be satisfied. Ideally, epidermis reconstructed from NHK should exhibit the anatomic and physiologic properties of natural skin extending from the proliferating basal cells at the dermal-epidermal junction through the normal pathway of terminal differentiation to generate the protective layers of the stratum corneum. Beside the biological properties, skin substitutes should exceed animal testing standards of availability, uniformity and cost effectiveness for common application.

In this report we characterize the histology of reconstructed epidermis on collagen-GAG populated with NHF and its response to two modulators of epidermal differentiation. Furthermore, we describe sustained proliferation of NHK in this model for 14 days.

Materials and Methods

Preparation of Collagen-GAG Substrates

Biopolymer substrates were prepared from bovine skin collagen and chondroitin-6-sulfate (GAG) as described previously [11]. Collagen and GAG were solubilized in 0.05 M acetic acid, mixed to form a coprecipitate, cast into sheets and frozen. The frozen precipitate was lyophilized, cross-linked by vacuum dehydration, and stored dry as a porous sponge. A non-porous film, that restricts NHK to one external surface in culture, was laminated onto the sponge, lyophilized and cross-linked as above. Dry substrates were rehydrated in 0.05 M acetic acid, cross-linked with 0.25% glutaraldehyde, washed repeatedly with purified water, and stored in 70% isopropanol. For cell culture, substrates were washed 3 X with phosphate-buffered saline (PBS) or HEPES-buffered saline, and 3 X with culture medium.

Cell-Substrate Composites, NHK and NHF

For differentiation studies, human skin obtained by plastic surgery was cut with a keratotome set at 0.4 mm. Split thickness skin was incubated overnight at 4 °C in a 0.25% (w/v) trypsin solution and the epidermis was separated from the dermis in a 0.05% (w/v) trypsin solution containing 0.02% (w/v) ethylenediaminetetraacetic acid (EDTA). After the addition of fetal calf serum to a final concentration of 50% (v/v) the cell suspension was gauze-filtered to eliminate the stratum corneum. The cells were recovered by centrifugation at 2,000 g for 5 min and grown with the 3T3 cell cocultivation technique [1]. For cell cycle studies, NHF were isolated using collagenase digestion as previously described, and cultured in serum-free MCDDB 153 [2].

NHF were isolated from dermal skin and cultured in Dulbecco's modified Eagle's medium (DMEM, containing 5.5 mM glucose and 4 mM glutamine) supplemented with 10% FCS. NHF were inoculated at a density of 3 X 10^6 cells/cm^2 onto the porous side of the collagen-GAG substrate. After 12 h, substrates with NHF were inverted to orient the nonporous surface up to seed the NHK. For differentiation studies, the NHK cultures were incubated with PBS containing 0.1% glucose (w/v) and 0.02% EDTA (w/v) to remove the remaining 3T3 cells. Thereafter, the keratinocytes were trypsinized, recovered by centrifugation, and seeded onto substrates at a density of 2.5 X 10^5 cells/cm^2.

Histology and Modulation of Epidermal Differentiation

For histology and modulation of epidermal differentiation, cell substrate composites were incubated in Dulbecco's modified Eagle's medium and Ham's medium F12 (1:1) containing 10% fetal calf serum, 100,000 units penicillin, 100 mg streptomycin, and 250 μg amphotericin B/liter of medium and were air-exposed on 0.5% agarose blocks (equilibrated with fresh culture medium) 1-4 days after seeding NHK. The NHK-NHF-collagen-GAG skin substitutes were kept air-exposed in a humidified incubator for an additional 7-14 days to promote optimal differentiation. The medium was changed every 2nd day.

To evaluate the effect of 25-hydroxycholesterol and sodium butyrate, two modulators of epidermal differentiation [12, 13], on the reconstruction of the
epidermis, the medium was supplemented with either 1 mg/ml 25-hydroxycholesterol or 1 mM sodium butyrate. After 14 days the samples were fixed in 10% phosphate-buffered formalin and embedded in paraffin; 5-μm sections were cut and stained with hematoxylin and eosin.

**Indirect Immunofluorescence Staining**

Frozen sections were air-dried and immunolabeled at room temperature by the indirect method. They were incubated for 30 min with the mouse anti-human particulate transglutaminase monoclonal antibody B.CI (obtained from Dr. S. Thacher, A&M College, College Station, Tex.). After washing in PBS, the samples were incubated with fluorescein-labeled anti-mouse Ig globulin (Dakopatts A/S, Denmark). They were washed in PBS and mounted in 90% (v/v) glycerol in PBS containing 5 mM p-phenylenediamine. The skin sections were observed with a Zeiss photomicroscope III.

**Cell Cycle Analysis of NHK Population Subsets**

NHK were isolated from epidermal skin with collagenase, and placed into primary culture in serum-free medium MCDB 153 supplemented with 10 ng/ml (epidermal growth factor), 5 μg/ml insulin, 0.5 μg/ml hydrocortisone, and 0.5 % bovine pituitary extract [2]. Before confluency, the cells were trypsinized, washed and recovered by centrifugation. They were inoculated at a density of 3 X 10^5 cells/cm^2 onto collagen-GAG substrates [9, 11]. Cell substrate composites were incubated submerged in serum-free medium MCDB 153, and medium was changed daily. Samples were removed after 1, 4, 7, 10, and 14 days of submerged incubation, NHK were isolated by treatment with dispase followed by trypsin-EDTA, and cell nuclei were prepared for flow cytometry [14]. Isolated cells in suspension were centrifuged (300 g). The pellet was washed with 5 ml PBS, centrifuged again, washed with 100 μg/ml cold PBS, 2 ml cold 70 % ethanol was added while vortexing, and incubated on ice for 30 min to fix cells. After fixation, cells were counted and incubated with RNase at a concentration of 600 μg/10^6 cells, 2 ml 1 N HCl were added to lyse the cells, and acid was neutralized with 1 ml 0.1 M Na_2B_4O_7, pH 8.4. Cell nuclei were centrifuged (600 g), resuspended in 2 ml 70% ethanol, and stored until all harvesting was completed. After all samples were collected, nuclei were labeled with propidium iodide (500 ng/ml) in PBS, filtered through Nitex fabric to remove debris, and 5 X 10^6 nuclei per sample were analyzed on a Becton-Dickinson FACStar. Excitation wavelength of analysis on the FACStar was 488 nm, and emission spectra were filtered through a FITC/PI filter assembly (band pass filters 530/30 and 620/35 in combination with a dichroic mirror 570). Samples were collected in triplicate at each time point from duplicate samples using separate NHK cell strains. Data are expressed as percent labeled nuclei (mean ± SEM) in G1, S, and G2 + M phases of the cell cycle.

**Results**

If human keratinocytes and fibroblasts are cocultured on a collagen-GAG biopolymer which is exposed to the air in a humidified incubator, an epidermis is reconstructed within 14 days that is very close to normal human epidermis in its histological appearance (fig. 1a compared to b). Particularly, the four major compartments of epidermis are seen in reconstructed epidermis. NHK attached to the collagen-GAG substrate are analogous to basal cells. Multiple layers of nucleated NHK are found above the 'basal' cells. The granular layer of reconstructed epidermis is at least as distinct as its counterpart in normal epidermis. Reconstructed epidermis also develops a desquamating stratum corneum with several layers that may provide physiologic barrier function. Although other anatomic features (pigmentation, immune effector cells, adnexal structures) of the epidermis are not seen, this reconstructed epidermis possesses the essential histological compartments of skin necessary for barrier function in vitro.

A comparative analysis of the distribution of the plasma membrane-associated enzyme transglutaminase in normal and reconstructed skin by indirect immunofluorescence staining revealed almost an identical
Fig. 1. Histological section prepared as described in Materials and Methods from normal human skin (a) and reconstructed skin (b) from NHK and NHF on a collagen-GAG biopolymer substrate.

distribution of the enzyme; however, in some areas of reconstructed skin, transglutaminase is already expressed in the lower spinous layers (fig. 2a compared to b). This finding is consistent with the development of a multilayered stratum corneum, and indicates that cornification in reconstructed epidermis is virtually normal.

If the medium contains 1 mM sodium butyrate, or 1 μg/ml 25-hydroxycholesterol, two modulators of epidermal differentiation [12, 13], the histological appearance of the reconstructed epidermis is altered (fig. 3a, b). In the presence of sodium butyrate (fig. 3a), which has been shown to be a potent inducer of morphological and biochemical cell differentiation [13, 15], disappearance of cell nuclei appears more pronounced in the lower layers of the reconstructed epidermis. Furthermore, no granular stratum develops, and the stratum corneum formed in the presence of butyrate has a more dense and compact appearance than without it. If the medium contains 25-hydroxycholesterol, many cells in the reconstructed epidermis remain parakeratotic and the stratum corneum is very thin (fig. 3b). 25-Hydroxycholesterol is known to reduce the formation of the corni-
fied envelopes [12], one of the major events in the process of terminal epidermal keratinocyte differentiation. There is also an absence of the granular stratum after exposure to 25-hydroxycholesterol.

Cell cycle analysis of NHK population subsets versus time of submerged culture in serum-free medium on collagen-GAG is shown in figure 4. One day after inoculation, the population of NHK in G1 decreases from 71.9 to 42.8%, with corresponding increase in the G2 + M population from 12.5 to 41.5% and no major shift in the S-phase population. Between day 1 and day 7, the G1 population increases from 42.8 to 72.4%, and remains at that level. The S-phase population increases from 15.7 at day 1 to 20.3% at day 4, decreases to 12.8% at day 7, and remains at that level through day 14. The G2 + M-phase population decreases from 41.5% at day 1 to 14.9% at day 7 and remains at that level through day 14. Between day 10 and day 14, all population subsets remain virtually constant, with cells distributed at about 73% in G1, 12% in S, and 15% in G2 + M.

Discussion

Reconstructed human epidermis can serve as a substitute for human skin in safety testing of drugs and consumer products [16], and in surgical grafting of deep skin wounds [17]. For safety testing, cultured skin must be compared to conventional standards of skin corrosion and irritancy (Draize skin test) [18], percutaneous absorption, and drug metabolism [19]. However, biological responses of reconstructed epidermis are measured by novel end points including cytotoxicity [20], release of inflammatory mediators and cytokines [21, 22], and epidermal penetration [19]. Therefore, correlation of these end points to existing standards is necessary to establish validity [23]. For wound closure, split-thickness skin restores the protective functions of skin, and defines the medical criteria for design of cultured skin substitutes [24]. Effective restoration of skin function depends on both reepithelialization of the wound, and on regeneration of connective tissue to replace the dermis, and to control scarring. The NHK-NHF-collagen-GAG...
Fig. 4. Plot of NHK percent labelled nuclei in the G1, S, and G2+M population subsets versus time in submerged culture on collagen-GAG substrates. Percent NHK in G1 shifts from 70% at the time of inoculation (day 0) to 40% after 24 h (day 1), increases from 40 to 70% by day 10 and remains constant until day 14. Cells in S-phase increase from 10% at day 0, to 20% at day 4, and then decrease to 15% by day 14. G2+M cells increase from 15% at inoculation to 45% at day 1, decreasing to 15% by day 10, and remaining constant until day 14.

Skin substitute described here provides the biological components necessary to establish new standards for toxicologic testing, and for surgery of deep skin loss injuries.

Comparison of reconstructed skin to natural skin demonstrates important similarities and differences. All epidermal strata are present, including the protective stratum corneum. Processes of epidermal differentiation including expression of the plasma membrane-associated transglutaminase, the enzyme responsible for the formation of cornified envelopes and lipid deposition, contribute to histological similarity of this cultured skin substitute to normal human skin. Restoration of stratum corneum is required for skin substitutes to develop properties of barrier function. Reestablishment of barrier function is of central importance to the use of cultured epidermis for standardized testing of percutaneous absorption and pharmacologic activities of topically applied drugs. Histological results of this study suggest lipid production and deposition in the stratum corneum of reconstructed epidermis, but a detailed lipid analysis has not yet been performed. Qualitative and quantitative analyses of lipid content of the stratum corneum are highly important to understand whether the barrier function and metabolism of pharmaceuticals in reconstructed skin are similar to normal skin.

Keratinocyte proliferation is demonstrated indirectly by development of a stratified and cornified epithelium. Cell cycle analysis shows a large shift of the NHK population into S-phase and cell division during the 1st week postinoculation onto collagen-GAG, followed by the establishment of a smaller population (15%) that continues to
replicate at a steady rate. Sustained keratinocyte replication leading to epidermal differentiation and reformation of the stratum corneum shows reestablishment in vitro of the dynamic equilibrium between proliferation and differentiation in human epidermis. Epidermal adnexal structures (hair, sebaceous and sweat glands) and immune effector cells (Langerhans cells, T cells, monocytes, neutrophils) of skin are absent in skin substitutes. Melanocytes are known to survive in cultured skin, but uniform melanization has not yet been demonstrated in vitro. The dermal component of cultured skin is also lacking microvascular endothelial cells and sensory function. Reconstruction of cultured skin substitutes with greater homology to normal skin requires greater understanding of the regulatory processes that occur in human skin in vivo, duplication of those processes in vitro, and inclusion of a full complement of cell types found in skin.

Advantages exist for the use of cultured skin substitutes in both toxicologic testing as an alternative to the use of animals, and in surgery of skin loss injuries from burns, dermatopathologies, and plastic surgery. For toxicology, greater numbers of tests can be performed at reduced cost per test, and greater biological definition and increased reproducibility of test results can be obtained. Furthermore, skin reconstructed from human cells eliminates extrapolation to humans of test results performed with animals. For surgery, cultured skin substitutes are more available, can reduce donor sites for split-thickness skin graft, reduce surgical operations, shorten hospitalization time, and produce superior outcome compared to split-thickness skin.

Human skin reconstructed in vitro represents the combined results from basic science studies in the fields of cellular, developmental and molecular biology, biochemistry, polymer chemistry, physiology, dermatology, oncology, surgery and other medical disciplines. The results obtained so far show that reconstructed skin consisting of human keratinocytes and fibroblasts cultured on collagen-GAG substrates gives rise to an epithelium that morphologically resembles healthy epidermis. The fact that a rather thick stratum corneum is present should be beneficial for its use as a closure of skin wounds as well as for studies to evaluate drug penetration in vitro. Furthermore, the responsiveness of the system to the two modulators of differentiation indicates that it could provide a practical, valid and available alternative for the establishment of new standards for in vitro pharmacology and toxicology.

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Dr. Rainer Schmidt
Centre International de Recherches Dermatologiques Galderma (CIRD Galderma)
Sophia Antipolis
F-06565 Valbonne Cedex (France)