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

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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 postinoculation. 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-

quirements 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 nonporous 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 $\times$ with phosphate-buffered saline (PBS) or HEPES-buffered saline, and 3 $\times$ 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 epi-dermis 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, NHK were isolated using collagenase digestion as previously described, and cultured in serum-free MCDB 153 [2].

NHF were isolated from dermal skin and cultured in Dulbecco's modified Eagle's medium (DMEM, containing 5.5 m$M$ glucose and 4 m$M$ glutamine) supplemented with 10% FCS. NHF were inoculated at a density of 3 $\times$ 10$^5$ 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 $\times$ 10$^5$ cells/cm$^2$.

For cell cycle studies, pure cultures of NHK were harvested by washing 2 $\times$ with HEPES-buffered saline, followed by trypsinization with 0.025% trypsin, 0.01% EDTA [2]. NHK were inoculated onto collagen-GAG substrates at a density of 3 $\times$ 10$^5$ cells/cm$^2$. Cell substrate composites for cell cycle studies contained NHK only, and no NHF.

### 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 μg/ml 25-hydroxycholesterol or 1 m$M$ 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.C1 (obtained from Dr. S. Thacher, A&M College, College Station, Tex.). After washing in PBS, the samples were incubated with fluorescein-labeled antimouse Ig globulin (Dakopatts A/S, Denmark). They were washed in PBS and mounted in 90% (v/v) glycerol in PBS containing 5 m$M$ 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 $3 \times 10^5$ 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/l 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$_2$B$_4$O$_4$, 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 fab-

ric to remove debris, and $5 \times 10^4$ 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
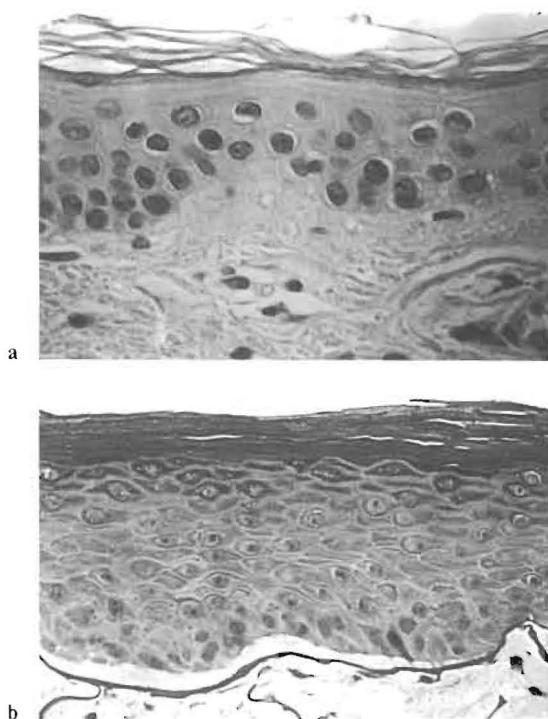
Fig. 1. Histological section prepared as described in Materials and Methods from normal human skin (a) and reconstructed skin (b) from NHK and NHF on a collagen-GAG biopolymer substrate.
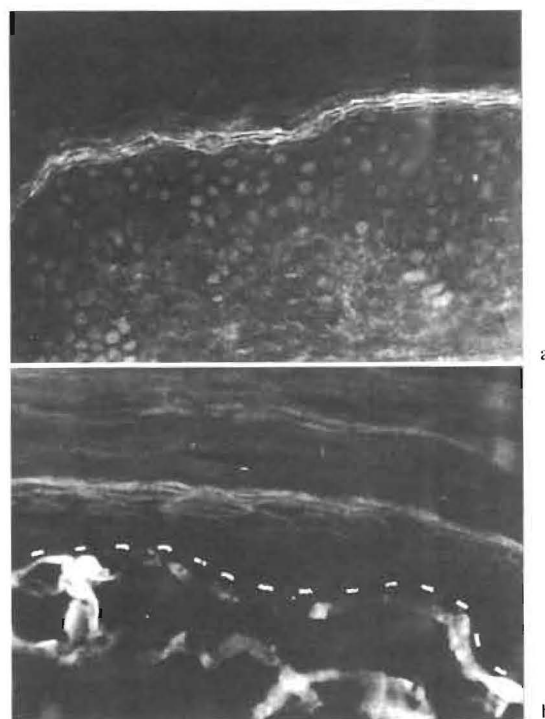
Fig. 2. Indirect immunofluorescence staining of the plasma membrane-associated transglutaminase in normal human skin (a) and reconstructed skin (b) from NHK and NHF of collagen-GAG biopolymer substrate.

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 m$M$ 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.



Fig. 3. Light microscopy histologies. a Reconstructed skin from NHK and NHF on collagen-GAG biopolymer substrate cultured in the presence of 1 m$M$ sodium butyrate. b Reconstructed skin from NHK and NHF on collagen-GAG biopolymer substrate cultured in the presence of 1 μg/ml 25-hydroxycholesterol.

## 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]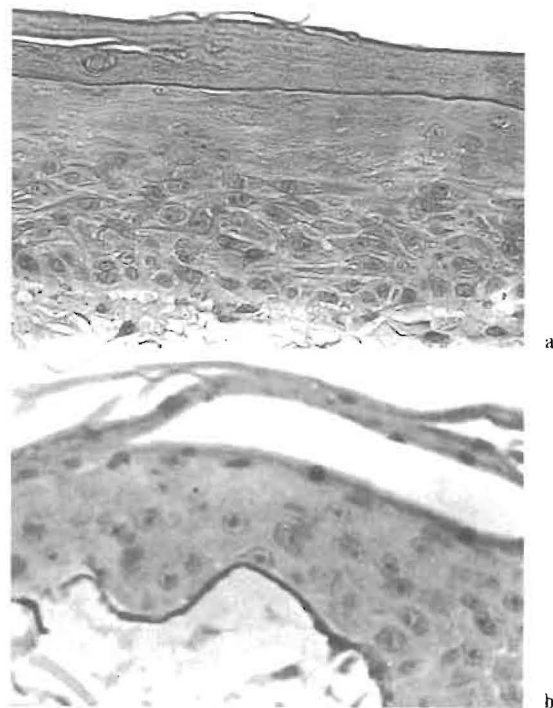, release of inflammatory mediators and cytokines [21, 22], and epidermal penetration [19]. Therefore, correlation of these end points to existing standards is necessary to establish validity [23]. For wound closure, split-thickness skin restores the protective functions of skin, and defines the medical 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
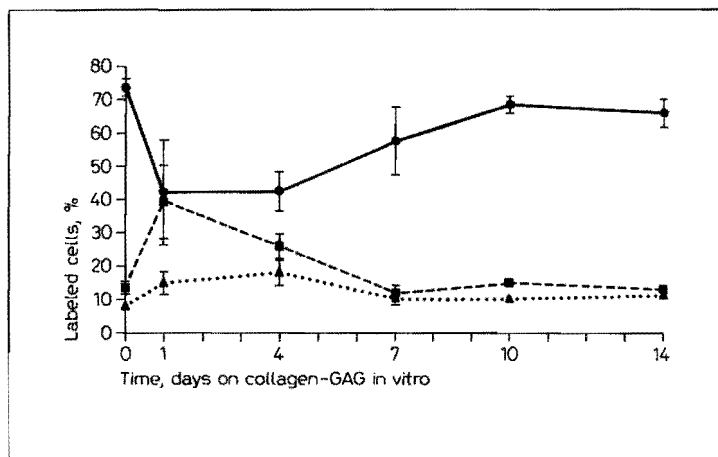
**Fig. 4.** Plot of NHK percent labelled nuclei in the G1, S, and G2+M population subsets versus time in submerged culture on collagen-GAG substrates. Percent NHK in G1 (●) shifts from 70% at the time of inoculation (day 0) to 40% after 24 h (day 1), increases from 40 to 70% by day 10 and remains constant 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 [25, 26] 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 standard-

ized testing of percutaneous absorption and pharmacologic activities of topically applied drugs [27, 28]. 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 keratino-
cyte replication leading to epidermal differ-
entiation and reformation of the stratum
corneum shows reestablishment in vitro of
the dynamic equilibrium between prolifera-
tion and differentiation in human epidermis.
Epidermal adnexal structures (hair, seba-
ceous and sweat glands) and immune effec-
tor cells (Langerhans cells, T cells, mono-
cytes, neutrophils) of skin are absent in skin
substitutes. Melanocytes are known to sur-
vive in cultured skin, but uniform melaniza-
tion has not yet been demonstrated in vitro.
The dermal component of cultured skin is
also lacking microvascular endothelial cells
and sensory function. Reconstruction of cul-
tured skin substitutes with greater homology
to normal skin requires greater understand-
ing 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, der-
matopathologies, 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 ob-
tained. Furthermore, skin reconstructed
from human cells eliminates extrapolation to
humans of test results performed with ani-
mals. 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 repre-
sents the combined results from basic
science studies in the fields of cellular, devel-
opmental and molecular biology, biochemis-
try, polymer chemistry, physiology, derma-
tology, 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 epi-
thelium 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 mo-
dulators of differentiation indicates that it
could provide a practical, valid and available
alternative for the establishment of new
standards for in vitro pharmacology and tox-
icology.

## References

1 Rheinwald JG, Green H: Serial cultivation of
  strains of human keratinocytes: The formation of
  keratinizing colonies from single cells. Cell 1975;
  6:337–343.
2 Boyce ST, Ham RG: Cultivation, frozen storage,
  and clonal growth of normal human epidermal
  keratinocytes in serum-free medium. J Tissue
  Cult Methods 1985;9:83–93.
3 Boyce ST, Ham RG: Calcium-regulated differen-
  tiation of normal human epidermal keratinocytes
  in chemically defined clonal culture and serum-
  free serial culture. J Invest Dermatol 1983;81/1:
  33s–40s.
4 Bell E, Ivarssen B, Merrill C: Production of a tis-
  sue-like structure by contraction of collagen la-
  tices by human fibroblasts of different prolifera-
  tive potential in vitro. Proc Natl Acad Sci USA
  1979;76:1274–1278.
5 Asselineau D, Prunieras M: Reconstruction of
  'simplified' skin: Control of fabrication. Br J Der-
  matol 1984;111:219–222.

6 Coulomb B, Lebreton C, Dubertret L: Regulation of fibroblast divisions in a human dermal equivalent culture model. J Invest Dermatol 1986;87: 134.

7 Lenoir MC, Bernard BA, Pautrat G, Darmon M, Shroot B: Outer root sheath cells of human hair follicle are able to regenerate a fully differentiated epidermis in vitro. Dev Biol 1988;130:610–620.

8 Prunieras M, Régnier M, Woodley D: Methods of cultivation of keratinocytes with an air-liquid interface. J Invest Dermatol 1983;81/1:28s–33s.

9 Boyce ST, Hansbrough JF: Biologic attachment, growth, and differentiation of cultured human keratinocytes on a graftable collagen and chondroitin-6-sulfate substrate. Surgery 1988;103: 421–431.

10 Hansbrough JF, Boyce ST, Cooper ML, Foreman TJ: Burn wound closure with cultured autologous keratinocytes and fibroblasts attached to a collagen-glycosaminoglycan substrate. J Am Med Ass 1989;262:2125–2130.

11 Boyce ST, Christianson D, Hansbrough JF: Structure of a collagen-GAG skin substitute optimized for cultured human epidermal keratinocytes. J Biomat Res 1988;22:939–957.

12 Ponec M, Kempenaar J, Weerheim A, Boonstra J: Differentiation of human keratinocytes: Changes in lipid synthesis, plasma membrane lipid composition, and $^{125}$I-EGF binding upon administration of 25-hydroxycholesterol and mevinolin. J Cell Physiol 1987;133:358–364.

13 Schmidt R, Cathelineau C, Cavey MT, Dionisius V, Michel S, Shroot B, Reichert U: Sodium butyrate selectively antagonizes the inhibitory effect of retinoids on cornified envelope formation in cultured human keratinocytes. J Cell Physiol 1989; 140:281–287.

14 Krishan A: Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. J Cell Biol 1975;66:188–193.

15 Leder A, Leder P: Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukemic cells. Cell 1975;5:319–322.

16 Goldberg AM, Frazier JM: Alternatives to animals in toxicity testing. Sci Am 1989;261:16–22.

17 Phillips TJ: Cultured skin grafts. Arch Dermatol 1988;124:1035–1038.

18 Draize JH, Woodard G, Calvary HO: Methods for the study of irritation and toxicity of substances applied topically to the skin and mucous membranes. J Pharmacol Exp Ther 1944;82:377–390.

19 Bronaugh RL, Maibach HI: In vitro percutaneous absorption; in Marzulli FN, Maibach HI (eds): Dermatotoxicology, ed 3. New York, Hemisphere Publishing, 1987, pp 121–133.

20 Norris DA, Ryan SB, Kissinger RM, Fritz KA, Boyce ST: Systematic comparison of antibody-mediated mechanisms of keratinocytes lysis in vitro. J Immunol 1985;135:1073–1079.

21 Sauder DN, Mounessa NL, Katz SI, Dinarello CA, Gallin JI: Chemotactic cytokines: The role of leukocytic pyrogen and epidermal cell thymocyte-activating factor in neutrophil chemotaxis. J Immunol 1984;132:828–832.

22 Luger TA, Kock A, Danner M, Colot M, Micksche M: Production of distinct cytokines by epidermal cells. Br J Dermatol 1985;113(suppl 28):145–156.

23 Boyce ST, Hansbrough JF, Norris DA: Cellular responses of cultured human epidermal keratinocytes as models of toxicity to human skin; in Goldberg A (ed): Alternative Methods in Toxicology. New York, Mary Ann Liebert, 1988, vol 6: Progress in in vitro Toxicology, pp 27–37.

24 Hansbrough JF, Boyce ST: What criteria should be used for designing artificial skin replacements and how do current grafting materials meet these criteria? J Trauma 1984;24:S31–S35.

25 Schmidt R, Reichert U, Michel S, Shroot B, Bouclier M: Plasma membrane transglutaminase and cornified envelope competence in cultured human keratinocytes. FEBS Lett 1985;186:201–204.

26 Schmidt R, Michel S, Shroot B, Reichert U: Transglutaminases in normal and transformed human keratinocytes in culture. J Invest Dermatol 1988;90:475–479.

27 Noonan PK, Webster RC: Cutaneous biotransformations and some pharmacological and toxicological implications; in Marzulli FN, Maibach HI (eds): Dermato-toxicology, ed 3. New York, Hemisphere Publishing, 1987, pp 71–93.

28 Dugard PH: Skin permeability theory in relation to measurements of percutaneous absorption in toxicology; in Marzulli FN, Maibach HI (eds): Dermato-toxicology, ed 3. New York, Hemisphere Publishing, 1987, pp 95–120.

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