Organotypic Culture of HaCaT cells: Use of Dermal Substrate that Combines de-epidermized Dermis with Fibroblast-populated Collagen Matrix

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Background **: The immortalized human keratinocyte line, HaCaT cells have been widely used as substitutes for normal epidermal keratinocytes. Recently, reconstruction of a skin equivalent using HaCaT cells showed a multilayered epithelium, but somewhat different tissue architecture as compared with normal epidermis.**

Objective **: In this study, using HaCaT cells we tried to reconstruct an epidermis resembling more closely to normal epidermis than the previous results.**

Materials and Methods: HaCaT cells were cultured in air-liquid interface on a recently developed dermal substrate in our laboratory, de-epidermized dermis (DED) raised on fibroblast-populated collagen matrix and the result was compared with those on DED or fibroblast-populated collagen matrix alone.

Results **: HaCaT cells on the new dermal substrate formed a multilayered epithelium with rete ridges, showing rather orderly cellular organization compared with those on fibroblast-populated collagen matrix. However, horny and granular layers were not observed contrary to normal epidermis. Immunohistochemical studies revealed that differentiation markers such as keratin 1, keratin 6 and involucrin showed the similar pattern to those in HaCaT cells cultured on fibroblast-populated collagen matrix. Markers of terminal differentiation, loricrin and filaggrin were not expressed contrary to normal epidermis.**

Conclusion **: These results suggest that organotypic culture of HaCaT cells on the dermal substrate combines DED with fibroblast-populated collagen matrix results in incomplete differentiation of HaCaT cells contrary to normal keratinocytes.**

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Key Words : HaCaT, DED, Fibroblast-populated collagen matrix, Differentiation

By culturing normal human skin keratinocytes on appropriate dermal substrates in air-liquid interface several skin equivalents have been developed. These skin equivalents provided multilayered epidermis closely resembling normal epidermis in vivo, thus have been used as tools not only to study skin biology but also skin pharmacology and stoxicology[1-3]. Of the skin equivalents two models, epidermis reconstructed on de-epidermized dermis (RE-DED) and epidermis on fibroblast-populated collagen matrix (living skin equivalent), have been widely used [4,5]. Recently, in our laboratory, by culturing keratinocytes on the new dermal substrate, which combined DED with fibroblast-populated collagen matrix epidermal differentiation was improved compared with RE-DED[6]. The use of normal keratinocytes as a material for skin

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equivalent, however, needs donor skin and has interindividual differences.

HaCaT cells are an immortalized keratinocyte line, derived from normal human abdominal skin[7]. They are independent of donor variations and available in unlimited quantity contrary to normal keratinocytes. HaCaT cells showed a differentiation profile comparable with normal human keratinocytes in submerged culture conditions[8]. Also, transplantation of HaCaT cells onto the subcutaneous tissue of athymic mice showed a nearly regular epidermal architecture[7,9]. Thus, they were widely used as substitutes for normal epidermal keratinocytes[10-12]. Recently, reconstruction of a skin equivalent using HaCaT cells was investigated in order to generate an alternative model to human skin[13-15]. However, in these studies, although HaCaT cells on DED or fibroblast-populated collagen matrix showed a multilayered epithelium, somewhat different tissue architecture as compared with normal epidermis was observed.

In this study, to reconstruct an epidermis resembling more closely to normal epidermis than the previous results, HaCaT cells were cultured in air-liquid interface on a recently developed dermal substrate in our laboratory, DED raised on fibroblast-populated collagen matrix and the result was compared with those on DED or fibroblast-populated collagen matrix alone.

MATERIALS AND METHODS

Cell culture

HaCaT cells, which were generously donated by Prof. Dr. NE. Fusenig (Heidelberg, Germany) and fibroblasts, which were derived from human foreskin during circumcision were grown in DMEM containing 10% fetal bovine serum (FBS). For organotypic culture fibroblasts were used at passages 4.

Dermal substrates

Fibroblast-populated collagen matrix was prepared as described previously[16]. Briefly, collagen solution mixture was prepared by quickly mixing eight volumes of collagen type 1 solution (Nitta Gelatin, Tokyo, Japan) with one volume of tenfold concentrated DMEM and one volume of sodium bicarbonate (22 mg/ml). Fibroblasts were added to the collagen solution mixture (1.5 mg/ml) at a concentration of 5×10^5 cells/ml. Aliquots (0.2 ml) of the fibroblasts and collagen solution mixture were plated into a 12-mm polycarbonate filter chamber (3.0 μm Millicell-pc; Millipore Co., Bedford, MA, USA) in six-well culture dishes.

DED, which was prepared as described previously[4], was placed into a Millicell or was raised on the fibroblast-populated collagen matrix in Millicell in six well culture dishes as described previously[6].

Organotypic culture of HaCaT cells

HaCaT cells (5×10^5 cells per Millicell) were seeded on top of DED or fibroblast-populated collagen matrix or DED raised on fibroblast-populated collagen matrix. They were cultured in submerged state for 2 days, and in air-liquid interface for 3 weeks. Cultures were maintained with growth medium consisting of a 3:1 mixture of DMEM and Ham's nutrient mixture F12 medium supplemented with 10% FBS, 5 μg/ml insulin, 1×10^{-10} M cholera toxin, 0.4 (g/ml hydrocortisone, 5 μg/ml transferrin, and 1×10^{-11} M triiodothyronine.

Histology and immunohistochemistry

In the growth period of 3 weeks in air-liquid interface, the samples were fixed in 4 % formaldehyde and embedded in paraffin for histology. Sections were stained with hematoxylin and eosin. Immunohistochemical studies were performed on deparaffinized sections using avidin-biotin-peroxidase complex technique (Dako, Carpinteria, CA, USA). Epidermal differentiation was investigated utilizing five antibodies: monoclonal antikeratin antibody 34β B4 (ENZO, New York, N.Y.) recognizing a 68-kDa keratin (keratin 1); monoclonal antibody to human keratin 6 (Novocastra, UK); polyclonal antibody to human involucrin (Biomedical Tech, Stoughton, MA); monoclonal antibody to human profilaggrin/filaggrin (Biomedical Tech, Stoughton, MA); and monoclonal antibody to human loricrin (Biomedical Tech, Stoughton, MA). To evaluate development of basement membrane zone, monoclonal antibody to human collagen IV (Dako, Carpinteria, CA, USA) was used.

RESULTS

Hematoxylin & eosin staining

HaCaT cells on DED formed a thin epithelium consisting of only one or two layers (Fig. 1a). HaCaT

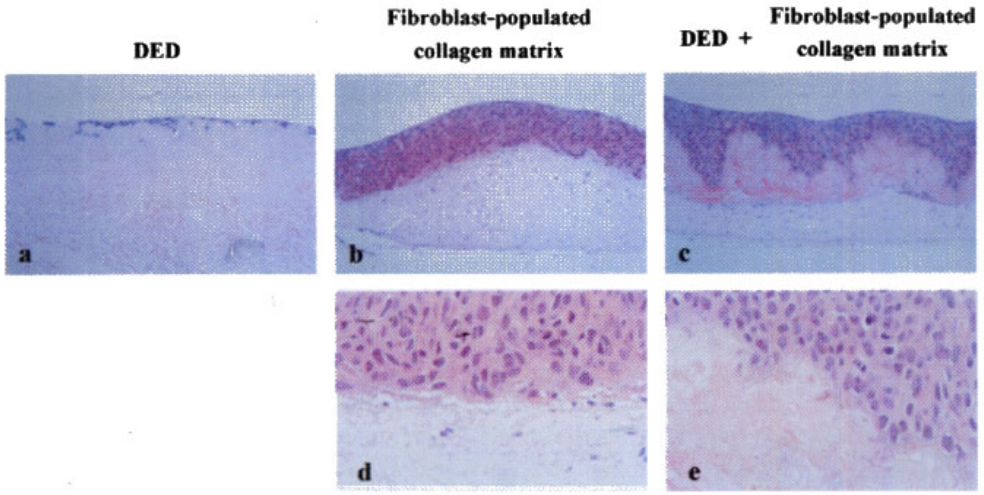

Fig. 1. Epithelial morphology of HaCaT cells cultured on (a) DED, (b) fibroblast-populated collagen matrix and (c) DED raised on fibroblast-populated collagen matrix for 3 weeks at the air-liquid interface(×100). High power view of lower portion of HaCaT epithelium on (d) fibroblast-populated collagen matrix and (e) DED raised on fibroblast-populated collagen matrix (×400). Hematoxylin/eosin staining.

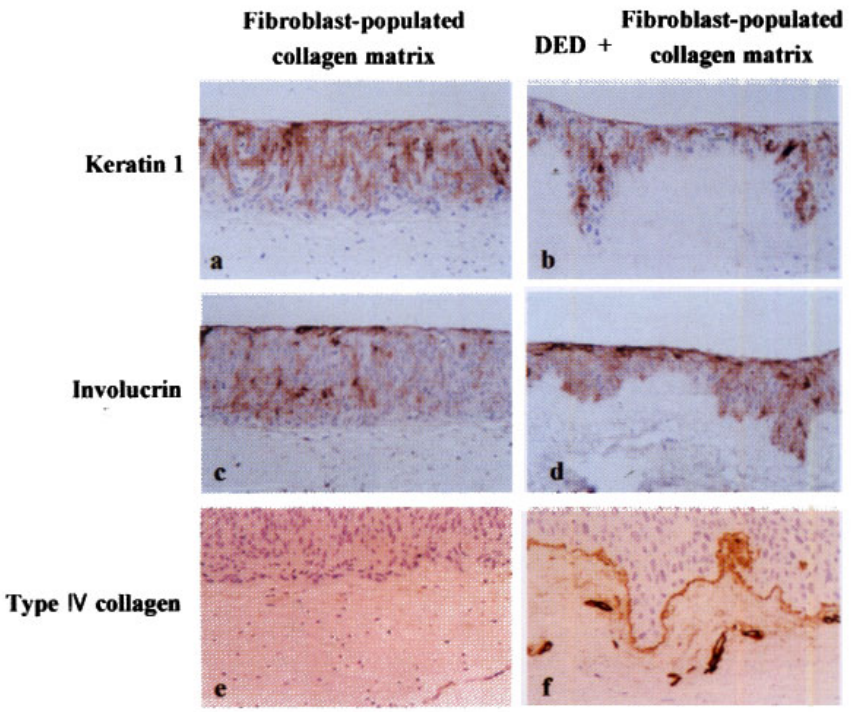

Fig. 2. Immunohistochemical staining of keratin 1(a, b), involucrin(c, d) and type Ⅳ collagen (e,f) in HaCaT epithelium on fibroblast-populated collagen matrix(a, c, e) and DED raised on fibroblast-populated collagen matrix (b, d, f) for 3 weeks at the air-liquid interface (×200).

cells on fibroblast-populated collagen matrix showed multilayered epithelium (Fig. 1b). When HaCaT cells were cultured on the new dermal substrate, that is, DED raised on fibroblast-populated collagen matrix they displayed better results (Fig. 1c) than those on fibroblast-populated collagen

matrix. Compared with HaCaT epithelium on fibroblast-populated collagen matrix (Fig. 1d) HaCaT epithelium on the new dermal substrate showed somewhat orderly cellular organization and rete ridges (Fig. 1e). However, similarly to HaCaT cells cultured on fibroblast-populated collagen matrix horny and granular layers were not formed contrary to normal epidermis.

Immunohistochemical study

Keratin: In HaCaT epithelium on fibroblast-populated collagen matrix keratin 1 was expressed irregularly in suprabasal layers (Fig. 2a). Also, when HaCaT cells were cultured on the new dermal substrate keratin 1 was expressed irregularly in suprabasal layers (Fig. 2b). Keratin 6, which is known as hyperproliferation marker, was expressed in all cell layers in both HaCaT epithelia on fibroblast-populated collagen matrix and the new dermal substrate (data not shown).

Involucrin: In HaCaT epithelium on fibroblast-populated collagen matrix involucrin was expressed mainly in suprabasal layers (Fig. 2c) similarly to HaCaT epithelium on the new dermal substrate (Fig. 2d).

Loricrin, Filaggrin: In both HaCaT epithelia on fibroblast-populated collagen matrix and the new dermal substrate loricrin and filaggrin were not expressed at all (data not shown).

Type Ⅳ Collagen: In HaCaT epitheium on fibroblast-populated collagen matrix type Ⅳ collagen was not found (fig. 2e). However, in HaCaT epithelium on the new dermal substrate type Ⅳ collagen was strongly expressed across epidermal-dermal junction and around blood vessels (Fig. 2f).

DISCUSSION

When human skin keratinocytes were cultured on appropriate dermal substrates in air-liquid interface multilayered epidermis with horny layer closely resembling normal epidermis in vivo was formed[4-5]. Also, by culturing normal oral keratinocytes in the same manner, multilayered epidermis without horny layer similar to oral mucosa in vivo was formed[16].

In this study HaCaT cells were cultured in air-liquid interface on a recently developed dermal substrate in our laboratory, DED raised on fibroblast-populated collagen matrix. HaCaT cells on the new dermal substrate formed a multilayered epithelium with rete ridges. However, horny and granular layers were not observed contrary to normal epidermis. Also, markers of terminal differentiation, loricrin and filaggrin were not expressed contrary to normal epidermis. These results suggest that differentiation of HaCaT cells on DED combined with fibroblast-populated collagen matrix is incomplete. Differentiation of HaCaT cells is thought to be incomplete because HaCaT cells are an immortalized keratinocyte line contrary to normal keratinocytes and culture conditions are different from the conditions in vivo.

When fibroblast-populated collagen matrix was added to DED or fibroblast-populated collagen matrix alone was used as a dermal substrate the layers of epithelium were much increased compared with HaCaT cells cultured on DED. This finding suggests that fibroblasts play an important role in stratification of HaCaT cells, consistent with the previous result[15].

By adding DED to fibroblast-populated collagen matrix as a dermal substrate, the morphology of HaCaT epithelium was slightly improved. In HaCaT epithelium on fibroblast-populated collagen matrix type Ⅳ collagen, a component of basement membrane was not found. On the contrary, in HaCaT epithelium on DED raised on fibroblast-populated collagen matrix type Ⅳ collagen was strongly expressed across epidermal-dermal junction These findings suggest that HaCaT cells are influenced by basement membrane in DED like normal keratinocytes[17,18]. However, when DED was added to fibroblast-populated collagen matrix epithelial differentiation did not seem to be improved compared with HaCaT cells cultured on fibroblast-populated collagen matrix alone.

In our laboratory, by culturing normal human keratinocytes on the new dermal substrate, which combined DED with fibroblast-populated collagen matrix multilayered epidermis with horny layer showing a similar morphology to that of native epidermis was made. Also, epidermal differentiation was improved compared with RE-DED[6]. However, even when fibroblast-populated collagen matrix was added to DED in HaCaT epithelium differentiation was incomplete. These results suggest that differentiation capacity of HaCaT cells in vitro were different from that of normal keratinocytes. Recently we found that in a skin equivalent HaCaT cells have a

preserved capacity to receive melanosomes but HaCaT cells do not have melanocytes to locate only in the basal location contrary to normal keratinocytes[19]. This finding may be related to incomplete differentiation of HaCaT cells.

There were three studies that HaCaT cells were investigated in order to reconstruct a skin equivalent similar to human skin[13-15]. In these studies as a dermal substrate fibroblast-populated collagen matrix, DED or fibroblast-populated DED were used. In our study in addition to DED, fibroblast-populated collagen matrix DED combined with fibroblast-populated collagen matrix was used as a dermal substrate. In one study HaCaT cells formed a well-structured and differentiated squamous epithelium, although an orthokeratotic keratinization was not achieved[15]. In the other studies, HaCaT cells showed multilayered epithelium but horny layer was not formed similarly to our results[13,14]. There were some differences between the results of previous studies and also that of this study. The different findings could be ascribed, most probably, to the use of different culture conditions including culture medium.

In conclusion, organotypic culture of HaCaT cells on the dermal substrate combines DED with fibroblast-populated collagen matrix results in incomplete differentiation of HaCaT cells contrary to normal keratinocytes. In order to use HaCaT cells as substitutes for normal epidermal keratinocytes further studies about culture conditions to improve differentiation of HaCaT cells will be needed.

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