Jpn. J. Cancer Res. 85, 238–244, March 1994

Growth and Differentiation Properties of Normal and Transformed Human Keratinocytes in Organotypic Culture

Makoto Tsunenaga,[1] Yohko Kohno,[2] Izumi Horii,[1] Shigeru Yasumoto,[3] Nam-ho Huh,[4] Tetsuhiko Tachikawa,[2] Shusaku Yoshiki[2] and Toshio Kuroki[4,5]

[1]Skin Biological Research Laboratories, Shiseido Research Center, Nippa-cho, Kohoku-ku, Yokohama 223, [2]Department of Oral Pathology, School of Dentistry, Showa University, Hatanodai, Shinagawa-ku, Tokyo 142, [3]Laboratory of Molecular and Cell Biology, Kanagawa Cancer Center Research Institute, Nakao-cho, Asahi-ku, Yokohama 241 and [4]Department of Cancer Cell Research, Institute of Medical Science, University of Tokyo, Shirokanedai, Minato-ku, Tokyo 108

The growth and differentiation of human normal keratinocytes and their transformed counterparts were examined in organotypic cultures in which the keratinocytes were grown at the air-liquid interface on top of contracted collagen gel containing fibroblasts. We developed a modified culture procedure including the use of a mixed medium for keratinocytes and fibroblasts. Normal keratinocytes formed a three-dimensional structure of epithelium that closely resembled the epidermis *in vivo*, consisting of basal, spinous, granular and cornified layers. Cells synthesizing DNA were located in the lowest basal layer facing the collagen gel. Expressions of proteins involved in epidermal differentiation were examined by immunohistochemical staining and compared with those in skin *in vivo*. In the organotypic culture, transglutaminase, involucrin and filaggrin were expressed, as in the epidermis *in vitro*, most prominently in the granular layer. Type IV collagen, a component of basement membrane, was expressed at the interface between the keratinocyte sheet and the contracted collagen gel. Keratinocytes transformed by simian virus 40 or human papilloma virus (HPV) exhibited a highly disorganized pattern of squamous differentiation. In particular, HPV-transformed cells invaded the collagen gel. Organotypic culture is unique in that regulatory mechanisms of growth and differentiation of keratinocytes can be investigated under conditions mimicking those *in vivo*.

Key words: Organotypic culture — Raft culture — Keratinocyte — Transformed keratinocyte — Differentiation

Isolation and cultivation of various types of cells maintaining their normal functions have been well developed. Keratinocytes, for example, can be isolated from skin and other squamous epithelia of humans and rodents, grown as monolayers in serum-free medium, e.g. MCDB153,[1)] and induced to differentiate terminally by various agents such as Ca^{2+} and vitamin D_3.[2,3)] Immortalization of human keratinocytes is achieved by introduction of oncogenic genes of DNA tumor viruses such as genes for the SV40[6] large T antigen, and E6 and E7 regions of HPV type 16 and 17.[4–7)]

Monolayer cultures are useful because they allow study of pure populations of cells under defined conditions. However, conditions in monolayer cultures differ from those *in vivo*, where there is homeostatic regulation of cell growth and differentiation under a rigid architectural organization. In skin *in vivo*, keratinocytes form a sheet of epidermis consisting of sequentially stratified layers from growing basal cells toward the terminally differentiated cornified layer. The surface of the epidermis is exposed to air while at the bottom, keratinocytes interact with the dermis through the basement membrane.

Organotypic culture of human skin has been developed as an approach to these conditions *in vivo*. In this system, keratinocytes are grown at the air-liquid interface on top of contracted collagen gel containing fibroblasts. The keratinocytes stratify and exhibit a typical pattern of differentiated squamous epithelium, generating a living skin model. In the present article, we describe the growth and differentiation properties of normal and transformed keratinocytes in the organotypic culture system currently used in our laboratory.

MATERIALS AND METHODS

Normal keratinocytes and fibroblasts Human epidermal keratinocytes were isolated from skin sections discarded during plastic surgery using a modification of the method reported elsewhere.[8)] Briefly, skin sections were cut into small pieces and incubated with 0.25% trypsin in PBS for 20–40 h at 4°C. The epidermis was separated from the dermis with fine forceps and keratinocytes were isolated

[5] To whom all correspondence should be addressed.

[6] Abbreviations: SV40, simian virus 40; HPV, human papilloma virus; PBS, phosphate-buffered saline; KGM, keratinocyte growth medium; DMEM, Dulbecco's modified Eagle's minimum essential medium; FCS, fetal calf serum; BrdU, bromodeoxyuridine; EGF, epidermal growth factor.

from the dermal side. Commercially available cultures of epidermal keratinocytes (Kurabo Co., Tokyo) were also used. Keratinocytes were grown in a modified serum-free KGM (Kyokuto Seiyaku, Tokyo) which consists of MCDB153 with high concentrations of amino acids, transferrin (final concentration, 10 μg/ml), insulin (5 μg/ml), hydrocortisone (0.5 μg/ml), phosphorylethanolamine (14.1 μg/ml) and bovine pituitary extract (40 μg/ml). The final concentration of Ca^{2+} in the medium was 0.03 m*M*. Subconfluent cultures of keratinocytes were dispersed with 0.025% trypsin and 0.02% EDTA in PBS. Soybean trypsin inhibitor was added at 2 mg/ml to stop the action of trypsin. Secondary cultures of the keratinocytes were used for organotypic culture.

Human fibroblasts were isolated from skin samples and grown in DMEM supplemented with 10% FCS. Subconfluent cultures were dispersed with 0.25% trypsin and 0.02% EDTA in PBS. Cells at population doubling levels of 10 to 15 were used for experiments.

Transformed keratinocytes Normal human keratinocytes were transformed by transfecting them with the HPV type 16 genome or the replication-defective SV40 genome as reported elsewhere.[9] In the present study, HPV-transformed PHK16II, its anchorage-independent derivative PHK16IISA-4 and SV40-transformed PSV1-1 were used. These cells were not tumorigenic when injected into nude mice.

Organotypic culture of human skin Organotypic cultures of human skin, often referred to as "raft cultures," were prepared according to the principles originally described by Bell *et al.*,[10-12] Pruniéras *et al.*[13] and Asselineau and Pruniéras.[14] As illustrated in Fig. 1, keratinocytes were cultured in a three-dimensional fashion at the air-liquid interface on top of a dermal equivalent consisting of type I collagen and fibroblasts.

For preparation of the dermal equivalent, one part of type I collagen of pig skin (Nitta Gelatin Co., Tokyo) and two parts of DMEM plus 10% FCS medium containing fibroblasts were mixed, while cooling, poured into an "untreated" 60-mm Petri dish at 10 ml/dish and allowed to gel at 37°C for 1 h. The final concentrations of collagen and fibroblasts were 1 mg/ml and 1×10^5 cells/ml, respectively. Contraction of the collagen gel was facilitated by tearing the gel from the surface of the Petri dish. For the first 7 days, DMEM plus 10% FCS was changed every 2 or 3 days, and was then replaced by KGM for another 1 day.

Keratinocytes were plated at $1.5\text{–}2.0\times10^5/cm^2$ inside a glass ring (8–12 mm diameter) on the surface of the contracted collagen gel, which was then placed on a stainless steel mesh. Keratinocytes were grown in KGM on day 1, and then in a 1:1 mixture of KGM and DMEM plus 10% FCS, in which the Ca^{2+} concentration was adjusted to 1.8 m*M*. The glass ring was removed on day 1.

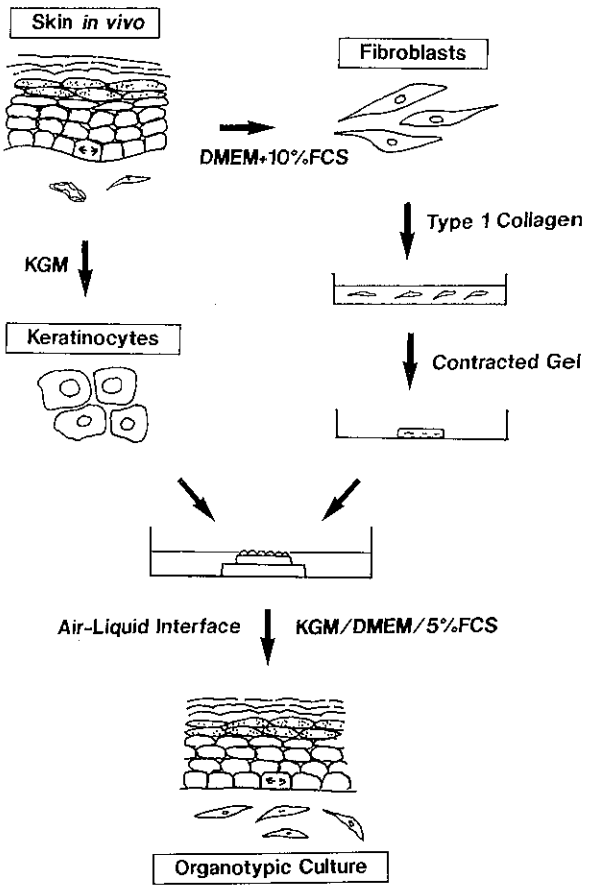

Fig. 1. Schematic illustration of organotypic culture of human skin. Keratinocytes are plated on top of contracted collagen gel containing fibroblasts and grow at the air-liquid interface.

The medium was added to the level of the keratinocyte sheet, so that the keratinocytes were grown at the air-liquid interface. The medium was changed every two or three days. Multilayered three-dimensional cultures of keratinocytes were obtained by day 7 to day 14.

Histological examination Organotypic cultures were fixed with 4% paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin and eosin. For immunohistochemical staining, the cultures were also fixed in O.C.T. compound (Miles Inc.) in liquid nitrogen and sectioned with a cryostat microtome.

Anti-human transglutaminase monoclonal antibody, anti-human involucrin polyclonal antibody and anti-human filaggrin monoclonal antibody were purchased from Biomedical Technologies Inc. (Stoughton, MA). Anti-human type IV collagen monoclonal antibody was reported elsewhere.[15,16] Reactivity was detected by a modification of the avidin-biotin immunoperoxidase

method. The specificity of staining was confirmed by use of non-immunized normal serum. The sections were counterstained lightly with hematoxylin.

Cells at the S phase were determined as those incorporating BrdU in 24 h and were detected using a kit (Amersham Co., UK) according to the manufacturer's protocol.

RESULTS

Contracted collagen gel Collagen gels containing fibroblasts contracted with time, from 12–24 h after the start of incubation, and the contraction reached a plateau after day 7. The rate of the contraction depended on the number of dermal fibroblasts seeded in the gel. With 10^6 cells per gel, a collagen gel of 10–15 mm diameter and 2–3 mm thickness was obtained after incubation for 1 week. The volume of the gel was estimated to be reduced to about 1/20–1/64 of the initial value, and so the contracted gel contained a high concentration of 20–64 mg of collagen per ml.

Formation of multilayered epidermis by normal human keratinocytes When plated on top of the contracted collagen gel and cultured at the air-liquid interface, normal human keratinocytes formed a multilayered epithelium by day 7–14 of organotypic culture. We used a 1:1 mixture of the medium for keratinocytes (KGM) and fibroblasts (DMEM+10% FCS) for the organotypic culture, since either one alone supported three-dimensional growth of keratinocytes only poorly. As shown in Fig. 2, the architecture of this cultured epithelium was very similar to that of normal human skin *in vivo* (see Fig. 4). Cells in the lowest layer attached to the collagen gel were polygonal and contained a relatively large, dense nucleus, like basal cells of the epidermis *in vivo*. Cells in the upper layers were flattened and contained a less prominent nucleus, corresponding to those in the spinous cell layer. The uppermost cell layer of the cultured epidermis was the granular cell layer: like those *in vivo*, the flat granular cells were tightly layered and contained many keratohyalin granules. The top of the multilayered epidermis was covered by the cornified layer. Occasional presence of nuclei in the cornified layer suggests that the cells entering this layer were not fully processed.

Location and frequency of cells synthesizing DNA Cells synthesizing DNA were detected by incorporation of BrdU. As shown in Fig. 3, cells with incorporated BrdU were localized in the lowest basal layer facing the collagen gel, and occasionally in the second layer. After exposure to BrdU for 24 h, this label was incorporated into 48% of the basal cells on day 6 and 5.6% on day 12.

Expression of differentiation markers Vertical sections stained with hematoxylin and eosin (Fig. 2) demon-

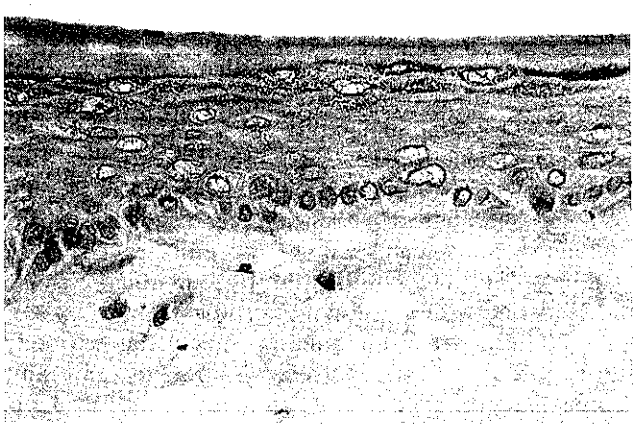

Fig. 2. Histological cross-section of an organotypic culture on day 12. Note that keratinocytes form a multilayered structure consisting of basal, spinous, granular and cornified layers. Hematoxylin and eosin stain.

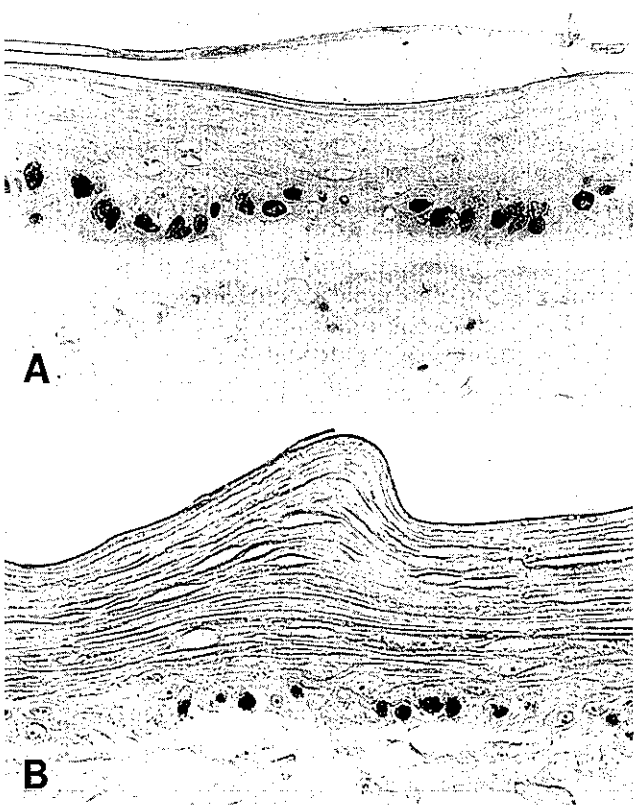

Fig. 3. Localization of cells with incorporated BrdU in the basal layer. Organotypic cultures on day 6 (A) and day 12 (B) were exposed to BrdU for 24 h and then stained immunohistochemically.

strated that the structure of the organotypic culture closely resembled that of the epidermis *in vivo*. Further comparison with the skin *in vivo* was made by immunohistochemical staining of various markers of epidermal differentiation. These markers were transglutaminase, involucrin, and filaggrin. Transglutaminase catalyzes ε-(γ-glutamyl)lysine cross-linking of protein precursors to form the insoluble cornified envelope, and so is a key enzyme of epidermal differentiation.[17] In normal skin *in vivo* (Fig. 4A), transglutaminase is expressed in the granular layer, whereas in organotypic cultures it was expressed in the granular layer and also in the upper one or two layers of the spinous layer (Fig. 4B).

Involucrin is one of the substrates of transglutaminase and is expressed only in the upper spinous layer and the granular layer in normal human skin *in vivo* (Fig. 4C).[18] In organotypic cultures, however, all the suprabasal layers right above the basal layer stained for involucrin (Fig. 4D).

Filaggrin is expressed in the granular layer of the epidermis *in vivo* (Fig. 4E) and it is thought to be necessary for the cross-linking of keratins that occurs during cornification.[19] In organotypic cultures, expression of filaggrin was detected in the granular layer and also the cornified layer, suggesting incomplete processing of filaggrin in the cornified layer (Fig. 4F).

Formation of basement membrane The formation of the basement membrane in organotypic cultures was examined by immunohistochemical staining of type IV collagen. Both in skin *in vivo* (Fig. 4G) and organotypic cultures (Fig. 4H), type IV collagen was present at the interface between the epidermal sheet and the dermis or the dermal equivalent.

SV40- and HPV-transformed keratinocytes We examined growth and differentiation properties of human keratinocytes immortalized by SV40 and by HPV type 16 in the organotypic culture. These cells are immortal and grow anchorage-independently, but are not tumorigenic when injected into nude mice.

When cultured on the contracted collagen gel, these transformed keratinocytes grew markedly differently from normal keratinocytes. As shown in Figs. 5 and 6, transformed cells did not form a stratified layer with upward sequential differentiation. They showed a highly disorganized pattern of squamous differentiation with no apparent granular and cornified layers. In particular, HPV-transformed cells invaded the collagen matrix and formed structures descending vertically from the surface (Fig. 6A). DNA-synthesizing cells, judged by incorporation of BrdU, were distributed throughout the cells invading the collagen gel (Fig. 6B). Type IV collagen was stained at the interface between SV40-transformed keratinocytes and the collagen matrix, but did not form a membranous structure (Fig. 5B).

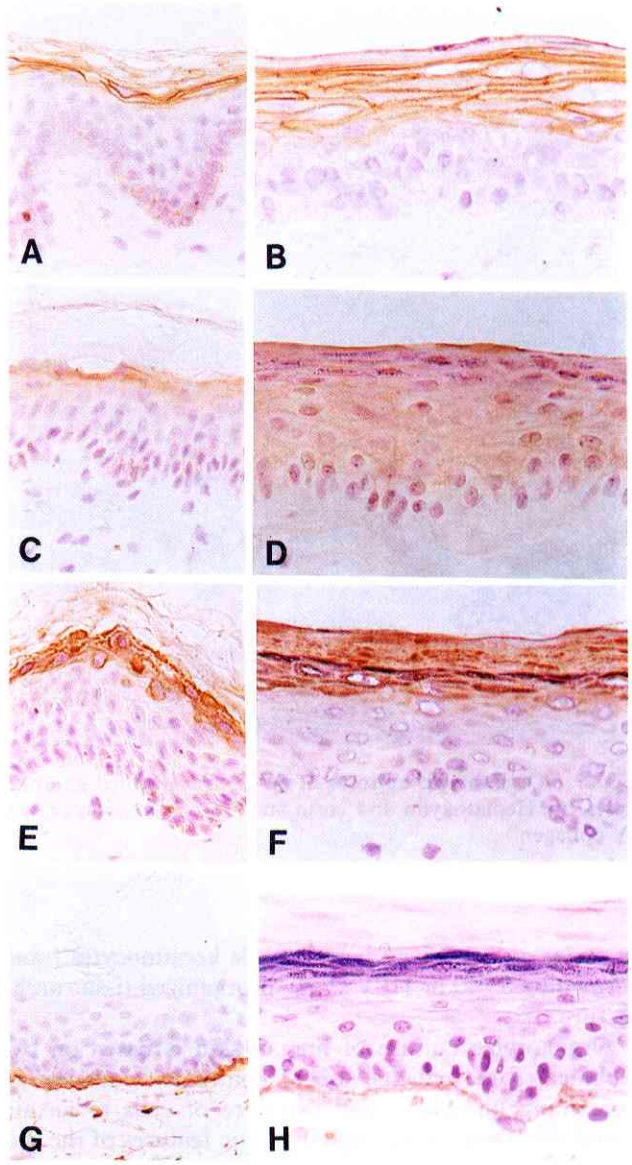

Fig. 4. Expression of differentiation markers and type IV collagen in organotypic cultures (right panels, i.e., B, D, F and H) in comparison with skin *in vivo* (left panels, i.e., A, C, E and G). These are transglutaminase (A, B), involucrin (C, D), filaggrin (E, F) and type IV collagen (G, H), and were detected by immunohistochemical staining. Light brown staining of the basal cells of skin *in vivo* is due to melanin pigments.

DISCUSSION

The present paper describes organotypic culture of human skin by reconstruction of epidermal keratinocytes and dermal fibroblasts. Normal keratinocytes form a stratified epithelial sheet consisting of basal, spinous,

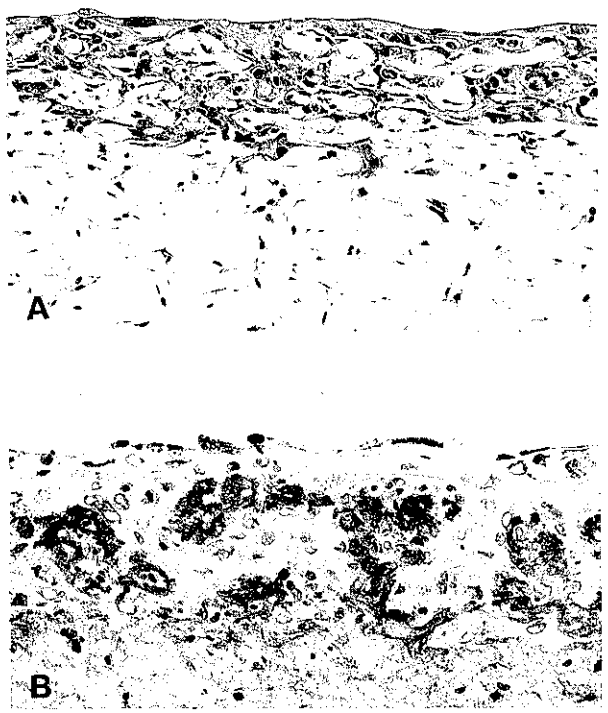

Fig. 5. Organotypic cultures of SV-40-transformed keratinocytes. A, Hematoxylin and eosin stain. B, Expression of type IV collagen.

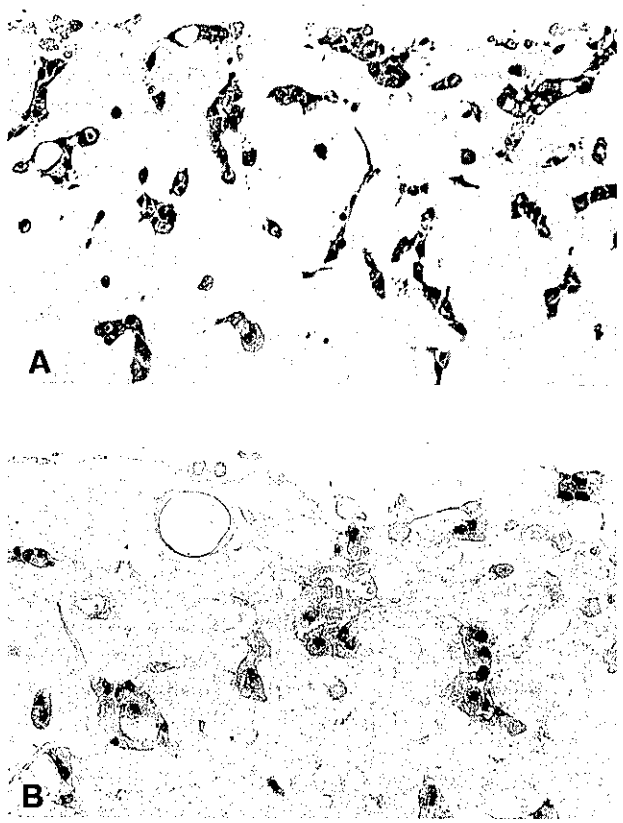

Fig. 6. Organotypic cultures of HPV-transformed keratinocytes. A, Hematoxylin and eosin stain. B, Distribution of BrdU-incorporated cells.

granular and cornified layers, while keratinocytes transformed by SV40 or HPV show disorganized tissue architecture.

Organotypic culture of human skin is based on two technical achievements, use of contracted collagen gel containing fibroblasts and exposure of cells to an air-liquid interface, both of which mimic features of the skin *in vivo*. Bell *et al.*[10-12] first reported contracted collagen gel prepared by seeding fibroblasts in the gel. Fibroblasts condense a hydrated collagen gel and produce a structure equivalent to the dermis. Human fibroblasts have been most commonly used for this purpose, but HPV-transformed keratinocytes have often been cultured with mouse 3T3 cells and without contraction.[20-26]

The second technical achievement of exposing keratinocytes to an air-liquid interface was made by Pruniéras and his colleagues.[13, 14] Under this condition, keratinocytes stratify and undergo terminal differentiation toward cornification. When submerged in culture medium, however, keratinocytes grow in a three-dimensional fashion but show incomplete cornification.

Thus, organotypic cultures are prepared by much more complicated procedures than monolayer cultures, but suitable conditions for each procedure have not yet been carefully examined. For example, the culture media used for most organotypic cultures were DMEM supplemented with FCS and some growth factors, e.g., EGF and transferrin.[27-37] Serum-free media have been developed for the monolayer culture of keratinocytes (e.g., MCDB153 and KGM), but these defined media have been used in only a few studies.[22, 38] In the present study, we used a 1:1 mixture of the media for keratinocytes (KGM) and fibroblasts (DMEM plus 10% FCS) for organotypic cultures: KGM alone supported the three-dimensional growth of keratinocytes only poorly. Our culture conditions do not allow maintenance of organotypic cultures for more than 3 weeks: on longer culture, all the keratinocytes undergo terminal differentiation, forming cultures consisting of a thick cornified layer. Thus, further improvements are needed for long-term maintenance of organotypic cultures.

Organotypic cultures of keratinocytes were reported to mimic the structure and functions of skin *in vivo* in

terms of the formation of the basement membrane,[32, 35, 36] the presence of proliferating cells in the basal layer and expressions of differentiation markers such as transglutaminase,[38] involucrin,[38, 39] filaggrin[24, 25, 38] and cytokeratins.[24, 25] In the present study, we also demonstrated the presence of these structural and functional markers. However, the expression patterns of the differentiation markers were not exactly the same as those in skin *in vivo*: these markers were not limited to the granular layer, but were expressed in broader layers, earlier or later in the process of squamous differentiation than in skin *in vivo*. These data suggest that the process of differentiation in organotypic cultures mimics that *in vivo* but is not exactly the same as the differentiation program *in vivo*, as pointed out by Asselineau *et al.*[39]

Organotypic culture has been used by researchers on HPV, who prefer to call it raft culture. Consistent with their reports, we found that HPV-transformed cells did not differentiate normally, showing disorganized patterns of growth and differentiation.[20–26] HPV-transformed cells penetrated into the collagen gel. These morphological characteristics closely resemble those seen in malignant cervical tumors *in vivo*. Recently, virions of HPV were found to be produced *in vitro* in organotypic cultures in close association with epidermal differentiation.[25, 26] Thus the infection program of HPV can now be investigated *in vitro* using organotypic cultures of keratinocytes.

Organotypic culture provides a unique method for study of many hypotheses on the structure and functions of epithelial tissues. Subjects that may be studied in the future using this system include paracrine growth factors that are possibly produced by epithelial-mesenchymal interactions, genes and gene products regulating the process of differentiation, structural and functional roles of the basement membrane and the localization of stem cells.

ACKNOWLEDGMENTS

This work was supported in part by a Grant-in-Aid for Special Project Research, Cancer-Bioscience from the Ministry of Education, Science and Culture of Japan.

(Received August 19, 1993/Accepted November 15, 1993)

REFERENCES

1) Boyce, S. T. and Ham, R. G. Calcium-regulated differentiation of normal human epidermal keratinocytes in chemically defined clonal culture and serum-free serial culture. *J. Invest. Dermatol.*, **81**, 33s–40s (1983).
2) Hennings, H., Michael, D., Cheng, C., Steinert, P., Holbrook, K. and Yuspa, S. H. Calcium regulation of growth and differentiation of mouse epidermal cells in culture. *Cell*, **19**, 245–254 (1980).
3) Hosomi, J., Hosoi, J., Abe, E., Suda, T. and Kuroki, T. Regulation of terminal differentiation of cultured mouse epidermal cells by 1α,25-dihydroxyvitamin D_3. *Endocrinology*, **113**, 1950–1957 (1983).
4) Rhim, J. S., Jay, G., Arnstein, P., Price, F. M., Sanford, K. K. and Aaronson, S. A. Neoplastic transformation of human epidermal keratinocytes by AD12-SV40 and Kirsten sarcoma viruses. *Science*, **227**, 1250–1252 (1985).
5) Dürst, M., Dzarlieva-Petrusevska, R. T., Boukamp, P., Fusenig, N. E. and Gissmann, L. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papillomavirus type 16 DNA. *Oncogene*, **1**, 251–256 (1987).
6) Pirisi, L., Yasumoto, S., Feller, M., Doniger, J. and DiPaolo, J. Transformation of human fibroblasts and keratinocytes with human papillomavirus type 16 DNA. *J. Virol.*, **61**, 1061–1066 (1987).
7) Münger, K., Phelps, W. C., Bubb, V., Howley, P. M. and Schlegel, R. The E6 and E7 genes of the human papillomavirus type 16 together are necessary and sufficient for transformation of primary human keratinocytes. *J. Virol.*, **63**, 4417–4421 (1989).
8) Kuroki, T., Hosomi, J., Munakata, K., Onizuka, T., Terauchi, M. and Nemoto, N. Metabolism of benzo(a)-pyrene in epidermal keratinocytes and dermal fibroblasts of humans and mice with reference to variation among species, individuals, and cell types. *Cancer Res.*, **42**, 1859–1865 (1982).
9) Yasumoto, S., Andoh, H., Kikuchi, K., Taniguchi, A., Hashida, T. and Takaoka, T. Altered growth regulation and immortalization of normal human epithelial cells induced by human papillomavirus type 16 oncogenes *in vitro*. *Tissue Cult. Res. Commun.*, **11**, 13–24 (1992).
10) Bell, E., Ivarsson, B. and Merrill, C. Production of a tissue-like structure by contraction of collagen lattices by human fibroblasts of different proliferative potential *in vitro*. *Proc. Natl. Acad. Sci. USA*, **76**, 1274–1278 (1979).
11) Bell, E., Ehrlich, H. P., Buttle, D. J. and Nakatsuji, T. Living tissue formed in vitro and accepted as skin-equivalent tissue of full thickness. *Science*, **211**, 1052–1054 (1981).
12) Bell, E., Sher, S., Hull, B., Merrill, C., Rosen, S., Chamson, A., Asselineau, D., Dubertret, L., Coulomb, B., Lapiere, C., Nusgens, B. and Neveux, Y. The reconstitution of living skin. *J. Invest. Dermatol.*, **81**, 2s–10s (1983).
13) Pruniéras, M., Régnier, M. and Woodley, D. Methods for cultivation of keratinocytes with an air-liquid interface. *J. Invest. Dermatol.*, **81**, 28s–33s (1983).
14) Asselineau, D. and Pruniéras, M. Reconstruction of 'simplified' skin: control of fabrication. *Br. J. Dermatol.*,

111 (Suppl. 17), 219–222 (1984).
15) Kino, J., Adachi, E., Yoshida, T., Nakajima, K. and Hayashi, T. Characterization of a monoclonal antibody against human placenta type IV collagen by immunoelectroblotting, antibody-coupled affinity chromatography, and immunohistochemical localization. *J. Biochem.*, **103**, 829–835 (1988).
16) Adachi, E., Kino, J., Hayashi, T., Sato, Y. and Hashimoto, P. H. The effect of fixatives and paraffin embedding on the histochemical reactivity of a monoclonal antibody against human type IV collagen (JK-199). *Arch. Histol. Cytol.*, **52**, 477–483 (1989).
17) Polakowska, R. R. and Goldsmith, L. A. The cell envelope and transglutaminases. *In* "Physiology, Biochemistry, and Molecular Biology of the Skin," 2nd Ed., ed. L. A. Goldsmith, pp. 168–204 (1991). Oxford University Press, New York and Oxford.
18) Eckert, R. L., Yaffe, M. B., Crish, J. F., Murthy, S., Rorke, E. A. and Welter, J. F. Involucrin–structure and role in envelope assembly. *J. Invest. Dermatol.*, **100**, 613–617 (1993).
19) Resing, K. A. and Dale, B. A. Proteins of keratohyalin. *In* "Physiology, Biochemistry, and Molecular Biology of the Skin," 2nd Ed., ed. L. A. Goldsmith, pp. 148–167 (1991). Oxford University Press, New York and Oxford.
20) McCance, D. J., Kopan, R., Fuchs, E. and Laimins, L. A. Human papillomavirus type 16 alters human epithelial cell differentiation *in vitro*. *Proc. Natl. Acad. Sci. USA*, **85**, 7169–7173 (1988).
21) Pecoraro, G., Morgan, D. and Defendi, V. Differential effects of human papillomavirus type 6, 16, and 18 DNAs on immortalization and transformation of human cervical epithelial cells. *Proc. Natl. Acad. Sci. USA*, **86**, 563–567 (1989).
22) Hudson, J. B., Bedell, M. A., McCance, D. J. and Laimins, L. A. Immortalization and altered differentiation of human keratinocytes in vitro by the E6 and E7 open reading frames of human papillomavirus type 18. *J. Virol.*, **64**, 519–526 (1990).
23) Blanton, R. A., Perez-Reyes, N., Merrick, D. T. and McDougall, J. K. Epithelial cells immortalized by human papillomaviruses have premalignant characteristics in organotypic culture. *Am. J. Pathol.*, **138**, 673–685 (1991).
24) Merrick, D. T., Blanton, R. A., Gown, A. M. and McDougall, J. K. Altered expression of proliferation and differentiation markers in human papillomavirus 16 and 18 immortalized epithelial cells grown in organotypic culture. *Am. J. Pathol.*, **140**, 167–177 (1992).
25) Meyers, C., Frattini, M. G., Hudson, J. B. and Laimins, L. A. Biosynthesis of human papillomavirus from a continuous cell line upon epithelial differentiation. *Science*, **257**, 971–973 (1992).
26) Dollard, S. C., Wilson, J. L., Demeter, L. M., Bonnez, W., Reichman, R. C., Broker, T. R. and Chow, L. T. Production of human papillomavirus and modulation of the infectious program in epithelial raft cultures. *Genes Dev.*, **6**, 1131–1142 (1992).
27) Bohnert, A., Hornung, J., Mackenzie, I. C. and Fusenig, N. E. Epithelial-mesenchymal interactions control basement membrane production and differentiation in cultured and transplanted mouse keratinocytes. *Cell Tissue Res.*, **244**, 413–429 (1986).
28) O'Keefe, E. J., Woodley, D. T., Falk, R. J., Gammon, W. R. and Briggaman, R. A. Production of fibronectin by epithelium in a skin equivalent. *J. Invest. Dermatol.*, **88**, 634–639 (1987).
29) Lillie, J. H., MacCallum, D. K. and Jepsen, A. Growth of stratified squamous epithelium on reconstituted extracellular matrices: long-term culture. *J. Invest. Dermatol.*, **90**, 100–109 (1988).
30) Madison, K. C., Swartzendruber, D. C., Wertz, P. W. and Downing, D. T. Murine keratinocyte cultures grown at the air/medium interface synthesize stratum corneum lipids and "recycle" linoleate during differentiation. *J. Invest. Dermatol.*, **93**, 10–17 (1989).
31) Schafer, I. A., Shapiro, A., Kovach, M., Lang, C. and Fratianne, R. B. The interaction of human papillary and reticular fibroblasts and human keratinocytes in the contraction of three-dimensional floating collagen lattices. *Exp. Cell Res.*, **183**, 112–125 (1989).
32) Chamson, A., Germain, N., Claudy, A., Perier, C. and Frey, J. Study of basement membrane formation in dermal-epidermal recombinants in vitro. *Arch. Dermatol. Res.*, **281**, 267–272 (1989).
33) Schürer, N. Y., Monger, D. J., Hincenberg, M. and Williams, M. L. Fatty acid metabolism in human keratinocytes cultivated at an air-medium interface. *J. Invest. Dermatol.*, **92**, 196–202 (1989).
34) Mak, V. H. W., Cumpstone, M. B., Kennedy, A. H., Harmon, C. S., Guy, R. H. and Potts, R. O. Barrier function of human keratinocyte cultures grown at the air-liquid interface. *J. Invest. Dermatol.*, **96**, 323–327 (1991).
35) Tinois, E., Tiollier, J., Gaucherand, M., Dumas, H., Tardy, M. and Thivolet, J. *In vitro* and post-transplantation differentiation of human keratinocytes grown on the human type IV collagen film of a bilayered dermal substitute. *Exp. Cell Res.*, **193**, 310–319 (1991).
36) Harriger, M. D. and Hull, B. E. Cornification and basement membrane formation in a bilayered human skin equivalent maintained at an air-liquid interface. *J. Burn Care Rehabil.*, **13**, 187–193 (1992).
37) Coulomb, B., Lebreton, C. and Dubertret, L. Influence of human dermal fibroblasts on epidermalization. *J. Invest. Dermatol.*, **92**, 122–125 (1989).
38) Rosdy, M. and Clauss, L.-C. Terminal epidermal differentiation of human keratinocytes grown in chemically defined medium on inert filter substrates at the air-liquid interface. *J. Invest. Dermatol.*, **95**, 409–414 (1990).
39) Asselineau, D., Bernard, B. A., Bailly, C., Darmon, M. and Pruniéras, M. Human epidermis reconstructed by culture: Is it "normal"? *J. Invest. Dermatol.*, **86**, 181–186 (1986).