Effect of Culture Matrix on Newborn Rat Skin Basal Cells; Modification of Cell Response to Extracellular Calcium

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Basal cells from newborn rat skin epidermis were cultured on various culture matrices, and the response of the cells to extracellular calcium was examined. When the cells were cultured on a type I collagen-coated Millipore filter to maintain clearance under the cultured cells as intact skin, the cells responded to a fluctuation in extracellular calcium and were detached from the collagen layer in a high calcium environment. In this case, protein synthesis of the cells increased calcium-dependently. In contrast, calcium-dependent protein synthesis of the cells was not detected in culture, neither on the plastic plate nor the type I collagen-coated plate. Moreover, in culture on a type I collagen-coated filter, the size of basal cells was unchanged for 2 d. This should be consistent with the fact that morphological changes in cells are very gradual in vitro. On the other hand, when basal cells were cultured either on a plastic plate or collagen-coated plate, the cells lost their normality; they were markedly enlarged for 2 d of culture.

Thus, the responses of skin basal cells to extracellular calcium fluctuated according to the types of culture matrices. In this case, keratin synthesis and a morphological change of the cells seems to be concerned with the clearance, which was established in the basal side of the cultured basal cells as intact skin.

Key words skin; calcium; basal cell; epidermal cell

The skin epidermal layer is constructed from various types of cells, such as basal, spinous, granular and cornified cells, and these cells are classified by their level of differentiation.1) Mechanisms of the formation of the epidermal layer have not been clarified. Recently, the peculiarity of intercellular calcium concentration has been reported in the skin epidermal cell layer,2) and it was suggested that calcium ions play an important role in the formation of the epidermal layer.3)

In epidermal (basal) cell culture, the culture matrix is one of the critical factors for the formation of the cell layer, because the attachment of basal cells to a culture substrate initiates the proliferation of the cells.4) Moreover, it is necessary to maintain the normality of basal cells in investigations of the effects of various materials on the cells. In a previous study, we obtained the cultured skin basal cell layer on collagen-coated Millipore filter, and examined the normality of the cell layer.5) This cell layer recognized endogenous low density lipoproteins, and transported them energy-dependently through an intracellular transport system,6) while the cell layer permeated extraneous nitrophenols through cellular junctions energy-independently.7) From these results we considered that the cultured basal cell layer on a collagen-coated filter was well maintained and expressed sensitivity to changes in extracellular environment.

As a first step in understanding the role of calcium, in this study we examined the effect of extracellular calcium on the proliferation of skin basal cells; i.e., their attachment to a supporting matrix, and the synthesis of keratins. Moreover, we discussed the correlation between the sensitivity of basal cells to calcium and the normality of the cells in various culture conditions.

MATERIALS AND METHODS

Reagents Reagents used in this study were as follows: Eagle’s minimum essential medium (MEM), Nissui Pharmaceutical Co., Ltd., Tokyo, Japan; fetal bovine serum (FBS), Whittaker M. A. Bioproducts, Walkersville, MD, U.S.A.; dispase, Godo-Shusei Co., Tokyo, Japan; Millicell-CM (12 mm diameter), Millipore Products, Bedford, MA, U.S.A.; type I collagen (bovine skin dermis), Koken, Tokyo, Japan; horse-radish peroxidase conjugated anti-rabbit IgG serum (goat), Cooper Biomedical, Malvern, PA, U.S.A.; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dojin-do, Kumamoto, Japan.

Preparation of Medium Eagle’s MEM was supplemented with 20 mm Hapes, 23.8 mm sodium bicarbonate and 10% FBS (MEM-FBS). MEM-FBS was treated with 2% Chelex 100 (Bio-Rad), and calcium levels in MEM-FBS were reduced by omitting Ca2+. This Chelex-treated medium was found to contain 0.01 mm Ca2+ by the Arsenazo III assay system.8) MEM-FBS, which contains several amounts of Ca2+, was prepared by the addition of CaCl2 to the Chelex-treated medium.

Preparation and Culture of Skin Basal Cells Basal cells were prepared from skin epidermis of 3-d-old Wistar rats by dispase and trypsin–EDTA digestion, as previously described.9) The cells were suspended in MEM-FBS, and seeded on a 24-well culture plate, a type I collagen-coated 24-well plate or a type I collagen-coated Millipore filter. The type I collagen-coated plate was prepared as follows: type I collagen was dissolved in acetic acid (3 mg/ml) and diluted with ethanol to 0.06% (w/v), 500 μl of the solution was applied to the well and dried overnight under ultraviolet (UV)-ray. Preparation of a collagen-coated filter has been previously described.9)

Measurement of Attached Cell Number After being

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cultured for an appropriate period, the culture medium was discarded with nonadherent cells. Then 0.5 μl of Ca$^{2+}$-, Mg$^{2+}$-free phosphate buffered saline (PBS) was added, and the adherent cells were gently dissociated from the culture matrix with a rubber policeman. The number of adherent cells was counted using a hemocytometer.

**Assay of Protein Contents in the Cells** After culture, a suspension of basal cells in 500 μl of PBS with 8 M urea was sonicated in a Branson Sonifier Cell Disrupter (model 185) at 0°C for 10 s. Protein content was determined by a Protein Assay Kit (Bio-Rad).

**Immunoblotting** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on 12.5% gel as described by Laemmli. Suspensions of basal cells in an appropriate volume of PBS with 8 M urea were sonicated in a Branson Sonifier Cell Disrupter (model 185) at 0°C for 10 s. The sonicates (10 μl) of the cells (2.0 × 10^4) were mixed with an equal volume of 0.5 M Tris–HCl (pH 6.8) containing 1% SDS, 650 mM 2-mercaptoethanol and 25% glycerol, boiled for 1 min, and then loaded on the gel. After electrophoresis, Western-blotting was performed as described by Towbin et al. Marker proteins used were: myosin (200 kDa), β-galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa) and ovalbumin (43 kDa).

**MTT Assay** Basal cells were cultured on a 96-well plastic plate (1.24 × 10^5 cells/well) or a type I collagen-coated Milipore filter (1.02 × 10^6 cells/well) in MEM-FBS with 1 mM MTT at 37°C for 4 h. After being cultured, 100 μl of 0.04 M HCl-99% isopropanol was added, and the formation of MTT-formazan was measured spectrophotometrically at 570 and 630 nm.

**RESULTS**

**Effect of Culture Matrix on the Response of Basal Cells to Extracellular Calcium** In the culture of basal cells on a culture plate, as shown in Fig. 1A, the attachment of

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**Fig. 1.** Effect of Extracellular Calcium on Attachment and Protein Synthesis of Basal Cells from Newborn Rat Skin

The cells were cultured on a plastic plate (A), type I collagen-coated plate (B) or type I collagen-coated filter (C) in MEM-FBS containing 0.05 mM (open circles) or 2.0 mM (closed circles) calcium at 37°C. The initial cell number is the number of seeded cells. Cells were cultured on a plastic plate (D; condition 1), collagen-coated plate (condition 2) or collagen-coated filter (condition 3) in MEM-FBS with 0.05 mM (hatched column) or 2.0 mM (closed columns) calcium at 37°C for 4 d. The amount of protein in the cells was determined with Coomassie brilliant blue G-250. Results are the mean ± S.E. of three determinations.

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**Fig. 2.** Micrographs of Newborn Rat Skin Basal Cells after Culture for 2 d at 37°C on Plastic Culture Plate

Basal cells were cultured on a plastic plate in MEM-FBS with 0.05 mM (A) or 2.0 mM (B) calcium. Bar, 10 μm.
the cells was suppressed by extracellular calcium, while protein synthesis of the cells was not affected by the concentration of calcium in the medium (Fig. 1D, condition 1). When the cells were cultured on a type I collagen-coated plate, the attached cell number decreased slightly and the protein content of the cells was almost unchanged by the concentration of extracellular calcium (Fig. 1B and D, condition 2). The culture systems, which used a plastic culture plate or type I collagen-coated plate, had no clearance in the basal side of the cultured basal cell layer. In skin, the dermal tissue exists under the epidermis and supplies nutrition to the basal cell layer through the basement membrane.12) Then we introduced a clearance in culture system of basal cells. As shown in Fig. 1C and D, condition 3, when the basal cells were cultured on collagen-coated filter, detachment and protein synthesis of the cells increased calcium-dependently.

Next we observed the morphological aspect of the cultured basal cell layer, which formed on various culture matrices. In the initial stage of culture, the size of basal cells was about 10 μm in diameter. When the basal cells were cultured with 0.05 mM calcium on a plastic plate for 2 d, the cells flattened to about 40 μm in diameter (Fig. 2A), and the size of the cultured cells did not change with incremental increases in calcium concentration to 2.0 mM (B). In the culture with 0.05 mM of calcium on a collagen-coated plate (Fig. 3A), the basal cells flattened to about 40 μm in diameter for 2 d. The size was significantly enlarged to 100 μm in diameter by increases in extracellular calcium (Fig. 3B). On the other hand, when the basal cell layer was formed on the collagen-coated filter with either 0.05 or 2.0 mM of calcium for 2 d, as shown in Fig. 4, the size of cells remained unchanged (10 μm). In this case, detachment of the cells from the collagen layer

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**Fig. 3.** Micrographs of Basal Cells after Culture for 2 d on Type I Collagen-Coated Plate

The cells were cultured on type I collagen-coated plate with 0.05 mM (A) or 2.0 mM (B) calcium. Bar, 10 μm.

**Fig. 4.** Micrographs of Cultured Basal Cells on a Type I Collagen-Coated Filter

Cells were cultured on a type I collagen-coated filter with 0.05 mM (A) or 2.0 mM (B) calcium for 2 d. Bar, 10 μm.
Fig. 5. Western Blots of Keratin of Newborn Rat Skin Basal Cells Cultured for 4 d

Basal cells were cultured on a plastic plate (A), type I collagen-coated plate (B) and type I collagen-coated filter (C) in MEM-FBS at 37 °C for 4 d. Anti-serum to keratin was used as the first antibody, and horse-radish peroxidase conjugated anti-rabbit IgG (goat) was used as the second antibody. Immunostaining was performed with 0.005% o-dianisidine (A) or 0.05% 4-chloro-1-naphthol (B and C). Lane 1, cells cultured with 2.0 mM calcium. Lane 2, cells cultured with 0.05 mM calcium.

Fig. 6. Effect of Extracellular Calcium on Formation of MTT-Formazan

A: In the presence of calcium (0.05-2.0 mM), basal cells were cultured on a 96-well plastic plate with 1 mM MTT at 37 °C for 4 h, and synthesized MTT-formazan was quantified spectrophotometrically at 570 and 630 nm. B: Cells were cultured on a type I collagen-coated filter with calcium (0.05 or 2.0 mM) and MTT (1 mM) at 37 °C for 4 h (hatched columns) or 8 h (closed columns). Results are the mean (± S.E.) of three determinations.

was observed by an increase in calcium concentration (Fig. 4A vs. B).

Effect of Extracellular Calcium on Activity of Basal Cells

Above, we described that a culture matrix influenced the protein synthesis of basal cells. Here, we examined whether extracellular calcium affects keratin synthesis of basal cells under various culture conditions. When the cells were cultured on a plastic plate or collagen-coated plate, three major bands (55, 60, 66 kDa) of keratin were detected, regardless of calcium concentration (Fig. 5A and B). In contrast, in the cultivation of basal cells on a collagen-coated filter, the cultured cells responded to extracellular calcium (C). In the presence of 2.0 mM of calcium, the bands were strongly stained (lane 1), while the bands were weakly detected by the cultivation with 0.05 mM calcium (lane 2). Moreover, calcium-dependent keratin synthesis was observed by immunofluorescence staining on the surface of the cultured basal cell layer, which formed on the collagen-coated filter (data not shown).

Next, we examined the activity of basal cells by MTT assay. The assay is dependent on a cellular reduction of MTT by the mitochondrial dehydrogenase of viable cells to a blue formazan product which can be measured spectrophotometrically. When the cells were cultured on a plastic plate for 4 h, as shown in Fig. 6A, the formation of MTT-formazan by the cells was independent of extracellular calcium. In the culture of basal cells on a type I collagen-coated filter, the production of MTT-formazan was significantly enhanced by extracellular calcium (Fig. 6B).

DISCUSSION

Recently, it has been reported that calcium plays an important role in the development and cell layer formation of skin epidermal cells. A calcium gradient is maintained in the intercellular space of the epidermis in vivo, where the basal and spinous cell layer are in a calcium environment below serum calcium levels, whereas
the granular cell layer and stratum corneum are in a high-calcium environment. The importance of this in vivo gradient in maintaining epidermal functions is suggested by in vitro studies of cultured murine keratinocytes, where the expression of either basal or suprabasal makers of differentiation is regulated by the extracellular calcium concentration is raised from 0.05 mM to levels above 0.1 mM in the culture medium. Since basal cells are located on the basement membrane and gain nutrition from the dermal side through the basement membrane in vivo, then we established clearance under the cultured basal cell layer in this study. When the basal cells were cultured on a plastic plate or collagen-coated plate, protein synthesis in the cells did not change with changes in extracellular calcium level. On the other hand, when basal cells were cultured on a collagen-coated filter, the cells responded to changes in extracellular calcium concentration, and protein synthesis increased calcium-dependently. Similarly, keratin synthesis and MTT-formazan formation was enhanced calcium-dependently only in cultivation on a collagen-coated filter.

In the skin, basal cells detach from the basement membrane and the shape of the cells gradually flattens, developing into the stratum corneum in about 30 d. In connection with these facts and the calcium gradient in the epidermis, intercellular calcium may regulate the detachment of basal cells from the basement membrane. Indeed, basal cells markedly detached from culture matrixes under a high calcium condition (2.0 mM) in this study. Furthermore, basal cells flattened and grew larger for only 2 d of culture in cultivation on a plastic plate or collagen-coated plate, but this is inconsistent with the fact that the development of basal cells to the flattened-stratum corneum takes 30 d in intact skin. From these findings we considered that the normality of basal cells was lost in cultivation on a plastic plate or collagen-coated plate. In contrast, basal cells seemed normal in culture on a collagen-coated filter, and the size of the cells was unchanged for only 2 d of culture.

Normality of the cells is one of the most important factors for an in vitro experiment. From this point of view, we considered that the effect of culture matrixes on the sensitivity and response of basal cells to extracellular calcium should be concerned with the normality of the cells. Next we will investigate the effect of culture matrixes on the normality of the cells by examination of the calcium-mediated keratin mRNA expression.

REFERENCES