Serum-Free Serial Culture of Adult Human Keratinocytes From Suction-Blister Roof Epidermis

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Coating cell culture flasks with natural extracellular matrix (ECM) enhanced the culture of adult human keratinocytes from suction-blister roof epidermis in an environment without fetal calf serum (FCS), bovine pituitary extracts or cellular feeder layers. A higher incidence of cell attachment on natural ECM was observed than on collagen and human fibronectins(HFN)-coated plastic dishes, and natural ECM was necessary for growth and proliferation of attached cells under the culture conditions used. Cells in primary culture grew to confluency on natural ECM-coated surfaces within about 14 days, and subsequent serial passage could be made up to fourth passage in collagen- and HFN-coated plastic flasks. Cultured keratinocytes in this serum-free environment formed colonies of small cuboidal, healthy cells with little keratinization or stratification and demonstrated antigenic characteristics of human basal cells.
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The suction-blister method is convenient and suitable to obtain the epidermis from infants and adults because it causes no pain and it produces no scar on the examined subjects. The roofs of the suction blisters are reported to be composed of pure epidermal cells lacking any dermal cells [1].

Although there have been many technical approaches to the culture of human keratinocytes, most culture methods need a cellular feeder layer and/or serum. The presence of serum or other cell types, however, prohibits the interpretation of certain types of experiments on cellular attachment or of protein synthesis, and may change the morphology and perhaps the biology of the cultured cells [2]. Recently, methods for serum-free culture of human keratinocytes have been reported [2–5], in most of which collagen and other matrix-coated growth substrata are used, or in which pituitary extracts (PE) replace fetal calf serum (FCS). Accumulating evidence on the biologic features of cultured keratinocytes in serum-free environments has provided new insights in basic investigative dermatology and some striking clinical applications.

It has been a long-range goal of many investigators to isolate and to grow human epidermal keratinocytes from both normal and diseased human skin in sufficient quantities for various biological studies [6]. This has not been feasible to date because most of the methods for keratinocyte culture have used neonatal foreskins. Recently, several investigators have reported methods of serial long term culture of adult keratinocytes: (1) from keratotome slices in media with FCS [6] or with PE (4); (2) from biopsy specimens in the medium with feeder layers [7]; (3) from suction blister epidermal roofs in medium with FCS [8].

The purpose of this study is to establish a more convenient method of serum-free and PE-free serial culture of adult human fresh keratinocytes from suction-blister roofs, since this method of obtaining epidermis is less damaging to the subjects, by using natural extracellular matrix (ECM)–coated flasks for primary culture.

MATERIALS AND METHODS

Suction-Blister Formation Skin specimens were obtained from 11 healthy volunteers (6 men and 5 women) ranging in age from 17 to 47 years with a mean of 33.5 years. The suction blisters were produced on the skin of the forearm using a suction device modified from the original apparatus described by Kiistala and Mustakallio [1]. The donor sites were warmed by portable lamp (100 W) at the distance of 50 cm during application of suction, and a negative pressure of 350 mmHg was applied through the suction apparatus. Within 1 h, blisters could be raised. From each volunteer, 3 blisters, each 4 mm in diameter, were obtained.

Primary Culture The roofs of the blisters were removed with a surgical blade and rinsed in solution A (a Ca^{2+}- and Mg^{2+}-free HEPE's buffered saline solution) containing antibiotics (100 IU/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml fungizon), then incubated in solution A containing 0.25% trypsin (Sigma, St. Louis, Missouri) at 37°C for 30 min. After incubation, the roofs were transferred into solution A with 10% FCS in the tube. Following gentle agitation, it was centrifuged for 7 minutes at 1400 rpm (Beckman TJ-6), the cell pellet was harvested, and the stratum corneum and supernatant were discarded. Epidermal cells were suspended in optimized nutrient medium MCDB153 (Lot # 906661004,6050483,6020202, Irvine Scientific, Irvine, California) supplemented with epidermal growth factor, insulin, hydrocortisone, ethanolamine, and phosphoethanolamine [3]. The stock

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Abbreviations:
FCS: fetal calf serum
ECM: extracellular matrix
HFN: human fibronectin
PE: pituitary extracts

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solution for primary culture and serial cultures consisted of MCDB153 supplemented with 0.1 mM Ca^{2+} plus elevated concentrations of amino acids (0.75 mM isoleucine, 0.24 mM histidine, 0.09 mM methionine, 0.09 mM phenylalanine, 0.045 mM tryptophan, and 0.075 mM tyrosine) [4]. The cells were plated at a density of 4×10^4 cells per cm^2 into a 25 cm^2 natural ECM-coated flask (Accurate Chemical & Scientific, Westburg, New York), which was kept at 4°C until use.

For the study of cell attachment, 4×10^4 cells per cm^2 were plated in 35 mm ECM-coated dishes (Accurate Chemical & Scientific) and 35 mm plastic dishes (Falcon, Lincoln Park, New Jersey) coated with collagen and human fibronectin (HFN). These methods for coating are described below. They were incubated at 35°C in humidified atmosphere containing 5% CO_2. The medium was changed after 24 h and thereafter every 2 days. The volume of medium was 5 ml/flask for the first week and then increased to 7.5 ml/flask.

Serial Culture Two- to 3-week-old primary cultures were incubated with sterile 0.05% trypsin and 0.01% EDTA in solution A for 1 min at 37° and 4 min at room temperature. The detached cells were suspended in solution A containing 20% FCS, and collected by centrifugation. The cell pellet was suspended in supplemented MCDB153 medium without FCS and transferred into 75 cm^2 flask (Corning, New York) coated previously with collagen and HFN.

Collagen and HFN Coating Collagen (Vitrogen, 3.1/mg/ml; Flow Laboratories, McLean, Virginia) was placed on the surface of a tissue culture plastic dish or flask (Corning) at concentrations of 1 ml/10 cm^2. Collagen in excess of the amount was removed by aspiration, and the surface rinsed with sterile 0.15 M NaCl containing antibiotics, followed by the second wash for 30 min in sterile 0.15 M NaCl. The coating of HFN was made according to Gilchrest and associates [2]. Briefly, HFN (Collaborative Research Inc., Lexington, Massachusetts) was added to the surface, previously coated with collagen, and incubated for at least 10 min at 35°C. Then, they were washed by sterile 0.15 M NaCl two times and conditioned at 35°C with supplemented MCDB153 medium for at least 1 h before use. As a control, some dishes were not coated.

Indirect Immunofluorescence (IF) Microscopy First-passage and second-passage keratinocytes grown for 4–6 days in a noncoated Lab-Tek culture chamber (Miles, Naperville, Illinois) were subjected to IF studies. The following antisera were used: rabbit antiserum to human keratin (DAKO, Santa Barbara, California), monoclonal antibodies (MoAb) for human skin basal cells (BC) (kindly given by Professor S. Imamura, Kyoto University) [9], MoAb to vimentin (DAKO), MoAb to Langerhans cells (OKT6, Ortho Pharmaceuticals, Raritan, New Jersey) and antiserum to melanocytes (S-100; Ortho Pharmaceuticals), FITC-conjugated goat anti–rabbit IgG (Cappel, Cochranville, Pennsylvania), FITC-conjugated goat $F(ab')_2$ anti–mouse IgG (Tago Inc., Burlingame, California). The detailed procedures were described previously [10]. At least 1000 cells were counted for each antibody tested. As for the positive control of the IF studies, the following cells or specimens were used: second passage melanocyte and fibroblasts obtained from neonatal foreskin of which culture methods were described elsewhere [11,12], and frozen section of suction blister epidermal roof. Staining was carried out according to the method of Huff and associates [13].

Others Cell attachment of epidermal cells was quantified visually by counting 20 random 0.6 mm^2 microscopic fields after gently washing twice with solution A and calculating the number of cells attached [14]. In some experiments, cultured dishes were gently washed three times with solution A, and keratinocytes were harvested in 0.05% trypsin and 0.01% EDTA [15]. Collected cells were counted by using a hemocytometer.

Table I. Effects of Surface Coating on the Attachment of Human Keratinocytes From Suction-Blister Roof

Coating	HFN Concentration	% Attachment
Collagen	0 $\mu g/cm^2$	44.5 (10.3)
	5	55.5 (8.4)
	10	49.4 (7.2)
Noncoating		18.3 (5.1)
ECM coating		63.2 (11.7)

The number is the percentage of keratinocytes attached to the culture dish 24 h after plating. Keratinocytes were obtained from the same volunteer (male, 34-years-old). The result in each condition is the mean (one standard deviation) of three separate experiments.

Cell viability was determined by trypan blue dye exclusion test.

Statistical analysis was made by Student's t test. Probability value over 0.05 was considered as insignificant.

RESULTS

A total of 36 primary cultures of adult keratinocytes obtained from dissociated suction blister roofs were performed using different media conditions and extracellular matrix conditions. Twenty-three of these primary cultures were performed on natural ECM-coated flasks. In initial experiments, cell attachment on different matrices was studied.

From 3 blister roofs (each 4 mm in diameter), $1.0–1.2 \times 10^6$ free epidermal cells could be consistently obtained; the viability of the epidermal cells was 61.5–75.0% (mean, 68.6%). One day after the start of the primary culture, cell attachment was determined by three separate experiments. As shown in Table I, the natural ECM-coated dishes showed the highest incidence of cell attachment and collagen- and HFN (5 $\mu g/cm^2$)-coated dishes showed relatively high attachment of epidermal cells. Table II shows the comparative time course of cell growth on natural ECM coated dishes and collagen and HFN (5 $\mu g/cm^2$) coated dishes, in which cells were harvested in trypsin and EDTA solution and counted by hemocytometer. A significant increase of cell number was observed in natural ECM-coated dishes, when compared with that in collagen and HFN-coated dishes. As another experiment, dissociated epidermal cells from 3 healthy individuals (2 men, 34- and 40-years-old; 1 woman, 33-years-old) were separately plated on natural ECM-coated, on collagen-coated, and on collagen and HFN-coated dishes. In the natural ECM-coated dishes, cells from 2 out of 3 volunteers grew to confluency in the serum-free media within 3 weeks. In dishes coated with other matrices, no cells from 3 volunteers would grow or proliferate to confluency. Based on these results, natural ECM-coated flasks (25 cm^2) were used for the majority of primary cultures of keratinocytes, and collagen- and HFN-coated flasks were used for the following serial culture.

On the first day of culture, keratinocytes attached on the surface of the flask were observed as small aggregates (Fig 1*a*). Six to 7 days after primary culture, the cells spread to form distinct colonies, which came to confluency within 7–10 days. Most of the cells in the confluency phase were small or cuboidal or elongated (Fig 1*b*). The number of differentiated cells was very small. In contrast, keratinocytes cultured in supplemented MCDB153 media containing 10% FCS showed flattened and irregular shapes with apparent differentiation and stratified (Fig 2). Contamination of fibroblasts could be found only when the negative pressure of the suction-blister device was over 400 mmHg. In some cultures, a few large colonies were observed and cells failed to reach confluency within 3 weeks. First passage was usually made 11 to 17 days (mean, 14.2 days from 20 separate experiments). Even if the cells were not confluent, subcultivation was carried out within 3 weeks because the number of stratified cells increased after day 20 if the cells were kept in primary culture. First-passage cells

Table II. Growth of Cultured Keratinocytes in Natural ECM Coated Dishes and Collagen- and HFN-Coated Dishes

Days after Plating	Dish Coating: Natural ECM	Dish Coating: Collagen + HFN ($5\mu g/cm^2$)	p Value
24 h	64.9 (11.8)	45.9 (12.7)	$0.05 < p$
5 days	53.1 (5.3)	35.5 (6.8)	$0.02 < p < 0.05$
7 days	58.2 (8.0)	34.3 (3.2)	$0.001 < p < 0.01$
9 days	68.1 (16.2)	38.8 (7.4)	$0.02 < p < 0.05$
11 days	75.5 (14.0)	40.2 (11.2)	$0.02 < p < 0.05$
14 days	86.1 (12.7)	48.0 (19.1)	$0.02 < p < 0.05$

The number is the percentage of keratinocytes (number of cells plated was estimated as 100%). Keratinocytes were harvested in trypsin and EDTA solution and counted by hemocytometer. The number in parenthesis was one standard deviation. Results at each time represent the mean of 3 or 4 separate experiments.

grew to confluency within 8 to 15 days (mean, 10.3 days from 14 separate experiments) after the first passage. First-passage cells grew slowly in the noncoated Lab-Tek chamber slide. The keratinocytes could be subcultivated up to 4 times but growth and proliferation of third-passage cells became very slow.

Twenty-three primary cultures of keratinocytes from suction blister roofs obtained from adult skin were performed on natural ECM-coated flasks using medium free from serum or PE. Successful serial cultures were achieved in 14 cultures, producing a success rate of 60.9%. During the same period as these experiments, serial culture of keratinocytes from neonatal foreskin was simultaneously carried out in supplemented complete MCDB153 with PE in noncoated plastic flasks [3], of which success rate was 45% (54/120). In the culture of keratinocytes from neonatal foreskin, the success rate was dependent on the lot number of commercial MCDB153 media used. The rate of success in our present study, however, was constant in different media lots.

Both first- and second-passage keratinocytes were identified by IF staining for keratin (100%) and vimentin (0%), and expressed basal cell antigens (99% IF staining with anti–basal cell MoAb) [Fig 3]. Melanocytes and Langerhans cells were not observed using S-100 polyclonal antiserum or OKT6 monoclonal antibody. All melanocytes and fibroblasts on Lab-Tek chamber slides showed positive reaction to anti–S-100 protein antisera and MoAb to vimentin, respectively. An appropriate number of OKT6 positive cells were demonstrated in the epidermis from which cell suspensions were prepared, but not in established keratinocyte cultures.

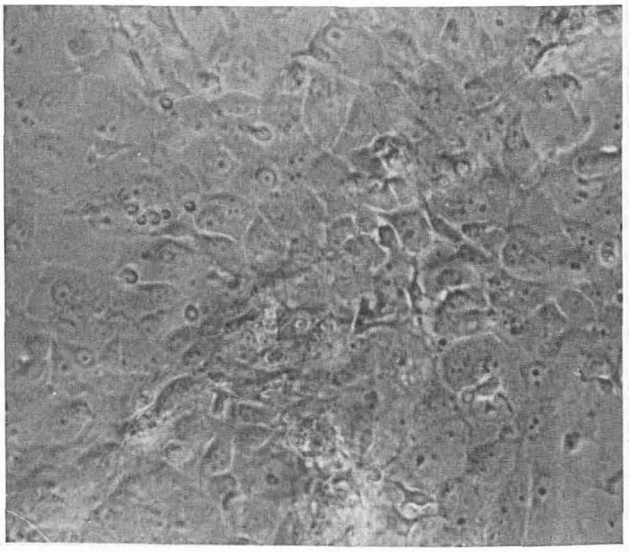

Figure 2. Phase contrast photograph of cultured keratinocytes (day 10) in supplemented MCDB153 medium containing 10% FCS (original magnification × 200).

DISCUSSION

In initial experiments, keratinocytes from suction-blister roofs were plated on the natural ECM-coated flask and cultured in the supplemented MCDB-153 medium with 10% FCS. The ability of keratinocytes to attach, proliferate, and endure subcultivation was studied as the use of FCS-supplemented medium was progressively decreased from the first 7 days, to 4 days, to one day. Finally, cells of fresh adult skin from suction-blister roofs could grow and proliferate on the natural ECM-coated surface in the serum-free, PE-free environment.

Cells of various origins, plated on the surface of tissue culture vessels and coated with a natural ECM produced by cultured corneal endothelial cells, are reported to undergo dramatic changes in their rate of attachment, morphologic appearance and growth characteristics [16,17]. Cells maintained on natural ECM had a higher growth rate and a lower serum requirement from those maintained on plastic. In addition, the rapid and firm attachment on natural ECM could restore the sensitivity of cells to physiologic factors [17] and rapid plating on natural ECM could reduce the damage effect of trypsinization [18,19]. These basic characters of natural ECM seem to be suitable for the culture of keratinocytes

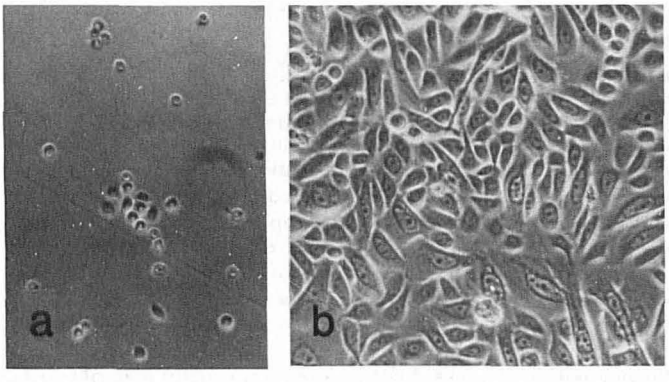

Figure 1. Phase contrast photograph of cultured keratinocytes in serum-free conditions. (a) 24 hours after plating, a small aggregate is seen (original magnification × 200). (b) Two weeks after plating cells reach confluence (original magnification × 200).

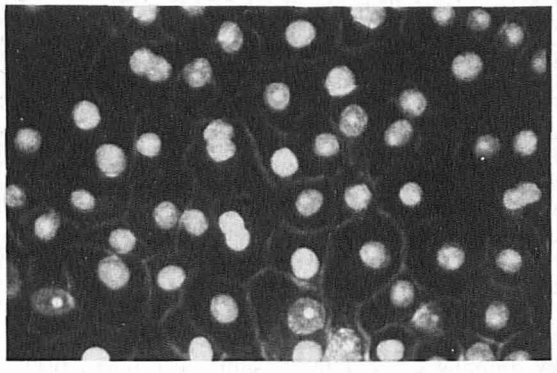

Figure 3. Indirect immunofluorescence photograph of the first passaged cells stained with monoclonal anti-basal cell antibody (original magnification × 400). Fluorescented second antibody staining is seen in an intercellular distribution. Nuclei are counterstained with propiodium iodide after fixation.

from suction-blister device, which might produce the damage of basal cells.

Previously, Kariniemi and colleagues [8] reported methods of culture of adult keratinocytes from suction-blister roofs. They used a MCDB medium containing 10% FCS, and cultured keratinocytes seemed to be highly keratinized. In contrast to their results, we were able to achieve attachment, proliferation and serial passage of small polygonal cells under serum-free conditions without stratification and differentiation.

Generally, serial cultures of adult epidermal keratinocytes have required a much higher number of primary plating cells (1×10^5–1.4×10^5 cells/cm^2) [6,8] than required of neonatal foreskin [3,15,16,20] because cells derived from neonatal foreskins may have a greater capacity for cell division in vitro [7]. The cell number (4×10^4/cm^2) in our present culture system seemed to be compatible with the previous reports [6,8]. Recently, Pittelkow and Scott [4] reported the success in the culture of adult keratinocytes obtained by a dermatome at a low density of 5×10^3/cm^2 in MCDB153 supplemented with PE but without FCS. Based on their report, 8 trial cultures of adult keratinocytes at the cell density of 5×10^3 cells/cm^2 were carried out using the present method, and two of them showed successful results (unpublished data). The discrepancy between their report [4] and our result may be due to the mechanical damage of basal cells by the suction-blister device or to the effects of PE.

Our method for culture of human adult keratinocytes allows one to perform experiments on epidermal cells for the study of cell-to-cell interaction, cell morphology, and secretion or production of biologic substances without the interference of serum or PE. Without the presence of serum, variation of Ca^{2+} concentration or of trace elements, such as strontium, can be used to modulate cell proliferation, level of differentiation, and perhaps function [21]. Importantly, these small colonies of cells with basal cell phenotype appear to be ideal targets for cytotoxicity experiments, using immunologic effectors suspected in vivo of damaging basal keratinocytes [22,23]. These techniques can be easily adapted to grow cells from individuals of different age, sex, and race and applied to patients with diseases of epidermal cell function or in whom epidermal keratinocytes are targets of immunologic damage. The high success made of primary cultures using this technique means that this is a practical method applicable to many different types of investigation in dermatology.

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