Human Epithelial Cells Induce Human Melanocyte Growth In Vitro but Only Skin Keratinocytes Regulate Its Proper Differentiation in the Absence of Dermis

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Abstract. Human keratinocytes isolated from a skin biopsy and cultured in vitro reconstitute a stratified squamous epithelium suitable for grafting on burned patients. Melanocytes coisolated from the same skin biopsy also proliferate under these culture conditions and maintain differentiated functions (i.e., synthesize melanin granules, regularly intersperse in the basal layer of the cultured epidermis, and transfer melanosomes in the cytoplasm of contiguous keratinocytes) (De Luca, M., A. T. Franzi, F. D'Anna, A. Zicca, E. Albanese, S. Bondanza, and R. Cancedda. 1988. Eur. J. Cell Biol. 46:176-180). Isolated melanocytes in culture grow in the presence of specific growth factors with a mean population doubling time of 4–10 d. In this paper we show that (a) human keratinocytes and oral epithelial cells possess strong and specific melanocyte growth stimulating activity (doubling time, 24 h); (b) melanocyte growth is not autonomous but requires close keratinocyte contact and is regulated to maintain a physiological melanocytes/keratinocytes ratio; and (c) pure skin keratinocytes, but not oral epithelial cells, have all the information required for the proper physiological location and differentiation of melanocytes in the epidermis.

Melanocytes are secretory cells uniquely located in the basal layer of the epidermis and are regularly interspersed among the basal keratinocytes in a structure defined as the epidermal–melanin unit (11). During fetal development, undifferentiated melanoblasts, arising from the neural crest, enter the skin via the dermis and inhabit the epidermis as a fixed cell population. The melanocyte/keratinocyte (M/K) ratio varies in different areas of the body and an average ratio of 1:36 has been reported in vivo (30). Melanogenesis represents the main biochemical function of the differentiated melanocyte. It has been shown that differentiated melanocytes undergo mitosis under physiological conditions (20), and a recent study has demonstrated that melanogenesis may be linked to the activation and subsequent proliferation of preexisting melanocytes (18). In mammals, melanocytes have been demonstrated in other tissues of the body such as the nervous system, uveal tract of the eye, inner ear, parathyroid gland, and heart; but in those tissues they are nonfunctioning and melanosomes transfer occurs only in the cytoplasm of epithelial cells (19). Human melanocytes have been isolated from skin biopsies and cultured in vitro. Phorbol esters (9), bovine brain extract (BEE, reference 31), basic fibroblast growth factor (16), and mitogenic factors present in extracts of melanoma, astrocytoma, and fibroblast cell lines (10), have been shown to be required for normal melanocyte growth in vitro. In these studies the cell population doubling time varied between 4–10 d (9, 10, 16, 31). Most of the known growth factors such as epidermal growth factor (EGF), nerve growth factor, transforming growth factor β (TGFβ), platelet-derived growth factor, interleukin 1α, substance P, and hormones such as insulin, hydrocortisone, triiodothyronine, and melanocyte-stimulating hormone, did not promote the growth of melanocytes in vitro (10, 16, 31).

Human keratinocytes isolated from a skin biopsy can be serially cultivated on a feeder layer of lethally irradiated 3T3 cells (25). Basal keratinocytes form colonies which grow at the periphery, start the differentiation process in their center, and eventually fuse giving rise to a stratified squamous epithelium suitable for grafting on burned patients (5, 12, 15).

It has long been known that keratinocytes and melanocytes can interact in culture (24), but little is known about the cellular and molecular mechanism of the cell–cell recognition and interaction. In a previous report, we have shown that melanocytes isolated from the skin biopsy will proliferate in the same culture conditions that allow keratinocyte growth

1. Abbreviations used in this paper: BBE, bovine brain extract; DOPA, 3,4-dihydroxyphenylalanine; EGF, epidermal growth factor; KGM, keratinocyte growth medium; MGM, melanocyte growth medium; M/K ratio, melanocyte/keratinocyte ratio; PMA, phorbol 12-myristate 13-acetate; TGF, transforming growth factor.
Materials and Methods

Epithelial Cell Culture

Human epidermal keratinocytes were obtained from skin biopsies and cultivated on a feeder layer of lethally irradiated 3T3-J2 cells (a gift from Dr. Howard Green, Harvard Medical School, Boston, MA), according to the method described by Rheinwald and Green (25). In brief, 2-cm² skin biopsies were minced and trypsinized (0.05% trypsin/0.01% EDTA) at 37°C for 3 h. Cells were collected every 30 min, plated (2 × 10⁷/75-cm² flask) on lethally irradiated 3T3-J2 cells, and cultured in 5% CO₂ in keratinocyte growth medium (KGM): Dulbecco-Vogt Eagle’s and Ham’s F12 media (3:1 mixture) containing 10% FCS, insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 μg/ml), cholera toxin (0.1 nM), triiodothyronine (20 pM), EGF (10 ng/ml; a gift from Dr. Carlos G. Nasimento, Chiron Corp., Emeryville, CA), and penicillin/streptomycin (50 IU/ml). Geneticin (100 μg/ml) was added for 2-4 d during plating and free-floating cells were removed. After 2-4 wk, wells containing single keratinocyte colonies were chosen. Cells were incubated in 0.02% EDTA for 10 min in order to obtain selective feeder-layer detachment; after pipetting, EDTA was aspirated and keratinocyte colonies were trypsinized. Cells from different clones were pooled, subjected to a DOPA reaction to verify the absence of melanocytes, and used in coculture experiments. In a typical experiment, 2 × 10⁶/cm² keratinocytes were plated in the presence or absence of lethally irradiated 3T3 cells together with 5 × 10⁶ melanocytes. Growth kinetics experiments were done in duplicate by counting total cells and DOPA-positive cells every 24-48 h up to confluence and stratification of the epithelium. Any modifications of these conditions are described in the Results section. In some experiments, the two cell populations were cultured in Transwell cell culture chambers (Costar Data Packaging Corp., Cambridge, MA) which allow rapid media diffusion without cell-cell contact.

Melanocyte Cultures

Normal human melanocytes were isolated from cultured, stratified, squamous epithelium obtained from skin keratinocytes in primary or secondary cultures. Confluent cultures were trypsinized (0.05% trypsin/0.01% EDTA) and the cell suspension was plated in the absence of feeder layer at a cell density of 2.5 × 10⁴/cm² in melanocyte growth medium (MGM): Dulbecco-Vogt Eagle’s and Ham’s F12 media (3:1 mixture) containing 5% FCS, insulin (5 μg/ml), transferrin (5 μg/ml), adenine (0.18 mM), hydrocortisone (0.4 mg/ml), cholera toxin (1 nM), triiodothyronine (20 pM), EGF (10 ng/ml), phorbol 12-myristate 13-acetate (PMA; 10 ng/ml), BBE (a gift from Dr. Enrico Albanese, IST, Genoa, Italy, prepared according to the procedure described by Maciag et al., [21]; 50 μg/ml), and penicillin/streptomycin (50 IU/ml). Although some of the above components (like EGF) were not strictly necessary to support melanocyte growth, we deliberately added them to the culture medium in order to minimize differences between MGM and KGM. Genetin (100 μg/ml) was added for 2-4 d during plating and the cells were passaged to avoid fibroblast contamination. 24 h after seeding, the medium was changed and free-floating cells were removed. After 2-4 d, melanocytes, identified by their morphology and reactivity to 1,3,4-dihydroxyphenylalanine (DOPA) (see below), were further purified by differential trypsinization (12). Melanocytes, preferentially detached at short trypsinization times (2-3 min), were collected in growth medium and plated at a cell density of ~5 × 10⁴/cm². Melanocytes were passaged at a 1:3 ratio and maintained in culture for >2 mo.

DOPA Reaction

Tyrosinase activity, a specific biochemical marker of the melanocyte, was assayed by the DOPA reaction according to the method of Becker et al. (2). To determine the ratio between melanocytes and keratinocytes in coculture experiments, cells were dissociated by trypsin, subjected to the DOPA reaction, and scored for positivity under the microscope. In some experiments sheets of cultured epithelium were detached from the surface of the vessel with neutral protease Dispase II (Boehringer Mannheim GmbH, Mannheim, FRG) (15), and then subjected to the DOPA reaction. Epithelial sheets were either spread upon a slide and viewed from the underside, or examined in vertical sections. To perform a DOPA reaction on attached cells, cultures were fixed in buffered formalin, the plasma membrane was permeabilized for 1 min in 0.1% Triton X-100, and the DOPA reaction was performed.

Mixed Cultures

Melanocyte cultures were obtained as described above. Pure epithelial cells were obtained by cloning procedures. Cells from confluent primary cultures were plated on lethally irradiated 3T3-J2 cells in six-well plates at a cell density of ~10-30 cells/well. After 2-3 wk, wells containing single keratinocyte colonies were chosen. Cells were incubated in 0.02% EDTA for 10 min in order to obtain selective feeder-layer detachment; after pipetting, EDTA was aspirated and keratinocyte colonies were trypsinized. Cells from different clones were pooled, subjected to a DOPA reaction to verify the absence of melanocytes, and used in coculture experiments. In a typical experiment, 2 × 10⁶/cm² keratinocytes were plated in the presence or absence of lethally irradiated 3T3 cells together with 5 × 10⁶ melanocytes. Growth kinetics experiments were done in duplicate by counting total cells and DOPA-positive cells every 24-48 h up to confluence and stratification of the epithelium. Any modifications of these conditions are described in the Results section. In some experiments, the two cell populations were cultured in Transwell cell culture chambers (Costar Data Packaging Corp., Cambridge, MA) which allow rapid media diffusion without cell-cell contact.

Results

Coculture Experiments

Human skin keratinocyte culture was established as described in Materials and Methods from biopsies obtained from different donors. In confluent primary cultures the M/K ratio varied from 1:30 to 1:77. To determine the growth rate of melanocytes and keratinocytes under these conditions, the cell populations were amplified 15-fold in secondary cultures. Cells were collected and counted at different time intervals and the M/K ratio was estimated by a DOPA reaction. The last M/K ratio was determined when a confluent stratified epithelium was obtained (6-8 d). As shown in Fig. 1, the growth rates of the two cell types were identical in all cell strains tested. During exponential growth, the mean cell population doubling time was ~24 h for both keratinocytes and melanocytes. This growth rate was maintained when keratinocytes were cultured in the absence of melanocytes (data not shown), whereas isolated melanocytes did not proliferate in the KGM (see below and Fig. 4). The M/K ratio estimated for each cell strain at the confluence was comparable to the M/K ratio in the parental cell population. Primary cultures were amplified 50-fold in order to investigate whether cell dilution was influential in sustaining melanocyte growth. Confluence was reached 1-2 d later compared to the 1:15 dilution but the doubling time for both cell types and the final M/K ratio were unchanged (Fig. 2, upper panels). A feeder layer of lethally irradiated 3T3-J2 cells was used for human keratinocyte growth and since cell extracts from WI-38, a fibroblast cell line derived from human embryonic lung, which has been shown to promote normal human melanocyte growth in vitro (10), to assay the role of 3T3 cells on melanocyte growth, the primary cultures were amplified 15-fold and seeded in the absence of a feeder layer. As shown in Fig. 2 (lower panels), both keratinocytes and melanocytes from
strain 89 and 90 proliferated with a doubling time of 24 h in the exponential growth phase but reached confluence later than cells grown in the presence of a feeder layer. Similar results were obtained analyzing two additional cell strains (not shown). Keratinocytes reconstituted a stratified squamous epithelium and in all cell strains the final M/K ratio was comparable to the ratio determined in the presence of the feeder layer (Fig. 2, lower panels). We could not perform the experiment at higher cell dilutions since under these conditions keratinocyte growth becomes feeder-layer dependent.

Confluent stratified epithelium in vitro behaves like normal epidermis in vivo (i.e., terminally differentiated cells are shed in the medium and replaced by basal keratinocytes induced to migrate in the upper layers). In confluent cultures, the basal keratinocyte doubling time is slower than in expanding colonies and is similar to the rate in the epidermis in vivo (13). In strain 89 and 90, the total number of melanocytes and the M/K ratio did not change when secondary cultures were examined 10 d after confluence (data not shown).

**Culture of Pure Cell Populations**

Epithelial cells from palatal biopsies can be isolated and cultured following the same procedure used for skin keratinocytes and the growth behavior is virtually indistinguishable. Oral epithelial cells reconstitute in vitro a stratified squamous epithelium successfully used in the treatment of large oral mucosa defects (De Luca, M., M. Megna, R. Cancr, P. Mangiante, and G. Sacco, manuscript submitted).
Figure 2. Growth kinetics of cocultured cells. Confluent primary cultures from strains 89 and 90 were trypsinized and plated (4 × 10^4/cm^2) on lethally irradiated 3T3-J2 cells (upper panels) or seeded (1.25 × 10^4/cm^2) in the absence of feeder layer (lower panels). Keratinocyte (solid circles) and melanocyte (open circles) growth was estimated as in Fig. 1.

for publication). Cells obtained from four oral palatal biopsies and the resultant in vitro cultured epithelium stained negatively for DOPA and melanocytes were absent in phase-contrast microscopy.

Human melanocytes were isolated from stratified cultured epithelium obtained from keratinocytes in primary and secondary cultures. As already reported, the absence of a feeder layer and the addition of PMA to the growth medium strongly inhibited keratinocyte growth and induced terminal differentiation of keratinocytes (9). High concentration of cholera toxin and the addition of geneticin selected against fibroblast proliferation (9). After two passages in the appropriate growth medium (see Materials and Methods), >99% of the cells were DOPA positive. Fig. 3 shows growth kinetics of isolated melanocytes in MGM (Fig. 3, solid squares) and in KGM (Fig. 3, solid circles). The doubling time in the exponential phase was ~72 h in the MGM (which contains PMA and BBE), whereas cells did not grow in the keratinocyte medium. Melanocytes never approached real confluence since dendritic processes touched and overlapped while overlapping of cell bodies was never observed (Fig. 4 a). In the early passages, phase-contrast microscopy revealed a mixed population of bipolar spindle-shaped cells with long, thin, and apparently connected dendritic processes (Fig. 4, a and b) and dark, star-shaped cells with large cytoplasm-rich melanosomes presenting several shorter dendritic processes also rich in melanosomes (Fig. 4 c). During cell passages in the presence of BBE and PMA, the relative amount of bipolar cells was increased and after seven to eight passages most of the cells in culture were spindle shaped. Star-shaped cells
Melanocytes

Figure 3. Melanocyte growth. Melanocyte culture was established as described in Materials and Methods. Purity of the culture was assayed by DOPA reaction (>99% positivity). Cells were plated in six-well plates (1 x 10^3/cm^2) in KGM (solid circles) or in MGM (solid squares). Melanocytes were collected, subjected to DOPA reaction, and counted every 3 d (mean of two experiments in duplicate).

readily reappeared when PMA was omitted from the culture medium. Melanogenesis was increased in the absence of PMA since star-shaped cell cytoplasm was highly enriched in stage IV melanosomes. Melanocytes were passaged at least 10 times at a 1:3 dilution without significant modifications in their growth behavior. Melanocytes obtained from five different keratinocyte cell strains have been cultured and no significant differences in their morphology were observed. However, the division time varied between 3 and 9 d.

Mixed Cultures

For the mixing experiments melanocytes passaged no more than three times were used. Melanocytes (strain M4M, 5 x 10^3) were plated in six-well culture plates together with autologous- and heterologous-cloned skin keratinocytes, oral epithelial cells, normal human fibroblasts (a gift from Dr. G. B. Ferrara, IST, Genoa, Italy), 3T3-J2 cells, and lethally irradiated 3T3-J2 cells (2 x 10^5). Except for autologous keratinocytes and 3T3, two different strains for each cell type were used. Cells were grown in KGM. After 7 d, cells were trypsinized and melanocyte growth was estimated by DOPA reaction (Fig. 5). Compared to control cells, viable 3T3 cells slightly stimulated melanocyte growth (fourfold) whereas heterologous keratinocytes, oral epithelial cells, and autologous keratinocytes strongly stimulated melanocyte proliferation (12-, 11-, and 12.6-fold, respectively). The final M/K ratios were 1:43, 1:42, and 1:27. Irradiated 3T3 and normal human fibroblasts did not promote melanocyte growth. All control cells plated in the absence of melanocytes were DOPA negative (not shown). Interestingly, a different morphology was noted between melanocytes grown with skin or palatal keratinocytes. Melanocytes grown with skin keratinocytes had a star-shaped polydendritic morphology whereas those grown with palatal-derived epithelial cells were mostly spindle-shaped. To test whether melanocytes were physiologically organized in the cultured epithelium obtained from heterologous keratinocytes and oral epithelial cells, confluent cultures were detached from the surface vessel with the neutral protease Dispase II and subjected to a DOPA reaction. No differences were noticed in the overall distribution of melanocytes when skin- and mucosa-derived epithelia were spread upon a slide and viewed from the underside (not shown). The distribution of the DOPA-positive cells was comparable to the distribution observed in autologous cultured epithelium (6). However, vertical sections of tissues showed melanocytes regularly interspersed between basal cells only in the epithelium cultured from skin keratinocytes (Fig. 6 a). In cultured oral epithelium, melanocytes were located throughout the epithelium layers, including the uppermost layers (Fig. 6 b, and inset).

To test if conditioned media from cultured epithelium were sufficient to promote melanocyte growth in the absence of M/K contact, skin keratinocytes, and melanocytes were cultured in two chambers of a cell culture device (Transwell, Costar Data Packaging Corp., Cambridge, MA) which allows media diffusion to physically separated cells. As shown in Fig. 7, growth was normally promoted by MGM but no significant melanocyte growth promoting activity was present in medium conditioned by keratinocytes (strain P6; Fig. 7,

Figure 4. Purified melanocytes in culture. Melanocytes (strain M4M) were isolated and cultured as described in Materials and Methods. Cells with overlapped dendritic processes and separated cell bodies are visible in a. Most of the growing melanocytes (in the presence BBE and PMA) had a spindle-shaped morphology (b), whereas freshly isolated melanocytes or cells grown in the absence of PMA had a star-shaped morphology (c). Bar, 100 μm.
Figure 5. Mixed cultures. Melanocytes (strain M4M) were cultured as described in Materials and Methods, and keratinocytes were cloned as described. After three passages, purified melanocytes were grown in KGM alone (A) or in coculture with lethally irradiated 3T3-J2 (B), 3T3-J2 cells (C), normal human fibroblasts (D), oral epithelial cells (strains M5 and M7; E), heterologous keratinocytes (strains P5 and P6; F), and autologous keratinocytes (G). Cells were plated on six-well plates at a cell density of 2 × 10^4/cm^2 and melanocytes were plated in a 1:40 ratio. After 7 d, cells were trypsinized and melanocyte growth was estimated by DOPA reaction.

When keratinocyte-conditioned medium was mixed with MGM no further stimulation of melanocyte growth was noticed. These results were confirmed by growing melanocytes in KGM containing 50% KGM previously conditioned for 24 h by actively growing keratinocyte colonies (not shown).

Figure 6. DOPA reaction on the cultured epithelium. Pure melanocytes (strain M4M) were cocultured with cloned skin keratinocytes (strain 95) and oral epithelial cells (strain M7) in a 1:40 ratio in secondary cultures. Epithelial cells were plated at a density of 2 × 10^4/cm^2. The confluent stratified epithelium obtained was detached from the surface vessel by the neutral protease Dispase II and subjected to DOPA reaction. Epithelial sheets were examined in vertical sections. a, skin keratinocytes; b, oral epithelial cells. Bar, 50 μm.

To establish if melanocytes had a clonal type of growth and localized in the basal layer of the cultured epithelium after confluence, or if keratinocyte colonies were associated with melanocytes from the beginning of the culture, strain M4M melanocytes were plated as above with autologous and heterologous keratinocytes (1:40 ratio). After 3 d, when keratinocyte colonies were evident in phase-contrast microscopy, DOPA reaction was performed on attached cells as described in Materials and Methods. In both cases ~80% of the melanocytes were found associated with keratinocyte colonies (Table I). At no stage of keratinocyte growth, did we ever observe keratinocyte colonies devoid of melanocytes.

Discussion

Normal human melanocytes are difficult cells to isolate and propagate in vitro since they represent only the 2–5% of the epidermis cell population and their growth rate in culture is very slow. Our experiments demonstrated that normal human epithelial cells possess strong melanocyte growth-promoting activity (mean cell population doubling time of 24 h). Skin keratinocytes isolated from a 2-cm^2 biopsy reconstitute 2 m^2 of stratified squamous epithelium in 3–4 wk (15) and, since cocultured melanocytes parallel keratinocyte growth, also melanocytes are amplified 10,000-fold in the same period. The M/K ratio in confluent cultures remained constant when cells were kept in culture up to 10 d after epidermis sheets reconstitution, suggesting that melanocyte growth and keratinocyte growth are coordinated. In agreement with the above finding is the observation that, already after 3 d of culture, >80% of the melanocytes were associated with actively growing keratinocyte colonies. The melanocyte growth-promoting activity of epithelial cells seems to be specific; normal human fibroblasts had no growth-stimulatory activity, and the slight stimulation by nonirradiated 3T3 cells fits previous data showing that extracts of cell lines from embryonic origin–induced (WI-38) melanocyte growth (10).

Recent studies have shown that cultured melanoma cells produce growth factors (4, 26) and a low molecular weight TGF which competes with EGF in binding assays has been...
Melanocytes associated with keratinocytes in the epidermis have been described in mammals in other tissues of the body and in particular in the mucous membranes. An analogous mechanism can be envisaged for melanocyte homing and organization in the epidermis. However, we cannot rule out the role of the dermis in driving melanocyte migration in the right location during fetal or postnatal development (experiments are in progress to test this hypothesis).

Melanocyte morphology has been correlated with its differentiation state. PMA has been shown to delay the onset of melaninization; and the cytoplasm of melanocytes grown in the presence of PMA contained much fewer melanosomes than melanocytes grown in cholera toxin alone (9). In addition, PMA induced a spindle-shaped morphology in cultured melanocytes whereas melanocytes grown in the absence of PMA were star-shaped (see reference 10 and Fig. 4). In both cases, the different level of differentiation did not preclude their replication. Our observation that melanocytes were mostly star-shaped when cocultured with skin keratinocytes and spindle-shaped when cocultured with oral-derived epithelial cells further suggests that only skin keratinocytes can induce proper melanocyte differentiation.

Melanocytes growing in the presence of PMA contained less melanin than melanocytes grown in cholera toxin alone (9). In addition, PMA induced a spindle-shaped morphology in cultured melanocytes whereas melanocytes grown in the absence of PMA were star-shaped (see reference 10 and Fig. 4). In both cases, the different level of differentiation did not preclude their replication. Our observation that melanocytes were mostly star-shaped when cocultured with skin keratinocytes and spindle-shaped when cocultured with oral-derived epithelial cells further suggests that only skin keratinocytes can induce proper melanocyte differentiation.

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De Luca et al. Keratinocytes Induce Melanocyte Growth

2195
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