Human sweat gland myoepithelial cells express a unique set of cytokeratins and reveal the potential for alternative epithelial and mesenchymal differentiation states in culture

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Summary

We have characterized precisely the cytokeratin expression pattern of sweat gland myoepithelial cells and have identified conditions for propagating this cell type and modulating its differentiation in culture. Rare, unstratified epithelioid colonies were identified in cultures initiated from several specimens of full-thickness human skin. These cells divided rapidly in medium containing serum, epidermal growth factor (EGF), and hydrocortisone, and maintained a closely packed, epithelioid morphology when co-cultured with 3T3 feeder cells. Immunocytochemical and immunoblot analysis disclosed that the cells differed from keratinocytes in that they were E-cadherin-negative, vimentin-positive, and expressed an unusual set of cytokeratins, K5, K7, K14, and K17. When subcultured without feeder cells, they converted reversibly to a spindle morphology and ceased K5 and K14 expression. Under these conditions, EGF deprivation induced flattening, growth arrest, and expression of α-smooth muscle actin (α-sma). Coexpression of keratins and α-sma is a hallmark of myoepithelial cells, a constituent of secretory glands. Immunostaining of skin sections revealed that only sweat gland myoepithelial cells expressed the same pattern of keratins and α-sma and lack of E-cadherin as the cell type we had cultured. Interestingly, our immunocytochemical analysis of ndk, a skin-derived cell line of uncertain identity, suggests that this line is of myoepithelial origin. Earlier immunohistochemical studies by others had found myoepithelial cells to be K7-negative. We tested five K7-specific antibodies that can recognize this protein in western blots and in the assembled keratin filaments of mesothelial cells. Three of these antibodies did not recognize the K7 present in myoepithelial cell filaments or in HeLa cell filaments, indicating that some K7 epitopes are masked when K7 pairs with K17 instead of its usual keratin filament partner, K19.

Key words: Cultured cell, Eccrine, Apocrine, Keratin, Actin

Introduction

The outer surface of the body is covered by epithelial tissues and structures that develop from the embryonic ectoderm. The epidermis, a stratified squamous epithelium, forms a protective barrier while the major adnexal structures of the skin, the hair and the sweat glands, provide the important function of regulating body temperature. Much has been learned about the biology of the epidermis and of the hair, including the kinetics of their renewal, factors that can modulate their growth, their pattern of cell division under normal and wound response conditions, and the major structural proteins responsible for their differentiated functions. The epidermis and the hair have been amenable to study in detail because these structures are so large and accessible, easy to examine biochemically and histologically, and because these tissues continually renew. In the case of the epidermis, the keratinocyte, which is the cell type that forms this tissue, can be grown in culture, permitting cell biologic, biochemical, and molecular genetic analysis of the mechanisms regulating its growth and differentiation.

Much less is known about the regulation of growth, tissue morphogenesis, and differentiation-related gene expression of the sweat glands. There are two types of sweat glands, eccrine and apocrine. Several million eccrine sweat glands having an estimated aggregate mass of about 100 grams are distributed over the body surface (reviewed by Sato, 1993). These glands are comprised of a coiled tube, lying at the border of the dermis and subcutaneous fat, connected to the surface by a duct that penetrates the epidermis to permit expulsion of the gland’s secretory products. The sweat glands can lower body temperature by producing a film of water on the surface of the skin that yields evaporative cooling. The secretory portion of the gland, at the distal end of the coil, transports an isotonic plasma filtrate into its lumen. A region of the gland farther along the coil, as it connects to the duct, contains absorptive cells which selectively remove NaCl such that the sweat is
hypotonic when it reaches the surface. Apocrine sweat glands form at different times and from a different cell lineage than the eccrine glands. Apocrine glands are formed as part of the pilosebaceous unit in several restricted regions, primarily in axillary and pubic skin, and they first become functionally active at puberty. They secrete sweat and other products (Cohn, 1994) into the pilosebaceous duct next to the hair shaft, rather than directly onto the epidermal surface. Unlike the epidermis and the hair follicle, eccrine and apocrine glands do not regularly renew themselves via cell division and terminal differentiation.

Histologic and immunohistochemical studies have revealed that eccrine glands begin forming at 13 weeks of gestation from clusters of cells that form buds in the embryonic epidermis and migrate downward as cords into the dermis. Initially, cells in the gland anlagen appear rather homogenous and express a combination of keratins and vimentin consistent with their identity as pluripotent progenitor cells which, as the gland matures, become restricted to one of three types of differentiation: duct, secretory, or myoepithelial (Holbrook and Wolff, 1993; Moll and Moll, 1992). Myoepithelial cells, a cell type defined by its expression of keratin-type intermediate filaments and α-smooth muscle actin (Franke et al., 1980; Norberg et al., 1992), surround and interdigitate with the secretory coil cells and are thought to provide tensile support against distention as the gland fills with sweat. These cells may also contract slightly in response to neurotransmitters, thereby aiding sweat expulsion (Sato et al., 1979).

Small, short-term cultures of ductal and secretory epithelial cells can be prepared from outgrowths of eccrine glands isolated by digesting skin specimens with dispase and collagenase and microdissecting the adnexal structures (see Collie et al., 1985; Lee et al., 1986; Jones et al., 1988; Yokozeki et al., 1990; Bell and Quinton, 1991). The culture of sweat gland myoepithelial cells has not yet been described, however. Scientists at a local biotechnology company who were serially culturing epidermal keratinocytes from skin biopsies to prepare grafts for burn patients (see O’Connor et al., 1981; Leigh et al., 1991) brought to our attention the occasional presence in these cultures of rare epithelial cell colonies of morphology distinct from that of keratinocytes. We report here the characterization of these as sweat gland myoepithelial cells and the identification of conditions promoting their rapid division in serial culture. We have found that this cell type expresses a unique pattern of cytokeratin proteins, including coexpression of the unusual K7/K17 cytokeratin pair, and that modifying culture conditions induces a reversible transition between epithelial and fibroblastoid morphology and pattern of cytoskeletal protein expression.

MATERIALS AND METHODS

Cells

Full-thickness skin biopsies of undamaged skin obtained from burn patients (typically from the axilla) were processed by the production laboratory at Biosurface Technology, Inc. (Cambridge, MA) as the first step toward expanding the keratinocyte population in culture to generate epidermal autografts (O’Connor et al., 1981; Leigh et al., 1991). The skin biopsies were minced into small fragments and stirred in 0.25% trypsin at 37°C. Released cells were recovered by centrifugation, resuspended in FAD medium, and co-cultivated with 3T3 feeder cells for ~7 days until cultures were nearly confluent. These primary cultures then were suspended with trypsin/EDTA and cryopreserved. Ampules were then thawed and plated under different conditions to expand the keratinocyte and myoepithelial cell populations.

Pure myoepithelial cell lines were isolated from cultures initiated from three skin biopsies: BRSO from the axilla of a 13-year-old (yo) male, CYHI from the axilla of a 28 yo female, and DOLA from the axilla of a 19 yo male. Mid-lifespan cultures of CYHI and BRSO were karyotyped by the Dana-Farber Cancer Institute’s clinical cytogenetics laboratory, using trypsin-Giemsa banding, and were found to be diploid female and male, respectively. Myoepithelial cells were also identified in, but were not isolated as pure populations from, cultures initiated from three other skin biopsies: JAKE from the abdomen of a 31 yo male, BABU from the axilla of a 28 yo female, and C5-SKH(m) from the scalp of a >40 yo individual whose gender was not recorded. The properties of myoepithelial cells in culture were compared with those of the normal newborn foreskin keratinocyte strain N (Rheinwald and Beckett, 1980), the normal human foreskin fibroblast strain S1-F, the human adult peritoneal mesothelial cell strain LP-9 (Wu et al., 1982), and the human skin-derived cell line ndk (Adams and Watt, 1988).

Culture conditions

Human myoepithelial cells and keratinocytes were routinely cultured in ‘FAD medium’, consisting of DME/F12 (Sigma) (1:1 v/v) + 5% newborn calf serum (Hyclone) + 5 μg/ml insulin, 0.4 μg/ml hydrocortisone (HC), 10 ng/ml epidermal growth factor (EGF), 1.8×10⁻⁴ M adenine, 10⁻¹⁰ M chola toxin, 2×10⁻¹¹ M triiodothyronine, and penicillin/streptomycin (Allen-Hoffmann and Rheinwald, 1984; Rheinwald, 1989). For use as feeder cells, the mouse fibroblast cell line 3T3 was grown in DME + 10% calf serum medium, either lethally irradiated with 5,000 R gamma-irradiation from a cobalt source or treated for 2.5 hours with 3 μg/ml mitomycin C (Sigma), and plated at ~5×10⁵ cells/cm² (Rheinwald and Green, 1975). Alternatively, myoepithelial cells were cultured in the absence of feeder cells in DME/F12 medium supplemented with 10% serum, hydrocortisone, and EGF. For some experiments, keratinocytes and myoepithelial cells were grown in Gibco keratinocyte serum-free medium (ker-sfm) (Life Technologies, Inc., Gaithersburg, MD) (Pirisi et al., 1987) supplemented with 30 μg/ml bovine pituitary extract, 0.1 ng/ml EGF, penicillin/streptomycin, and additional CaCl₂ to bring the total [Ca²⁺] to 0.4 mM (Schön and Rheinwald, 1996). Ndk cells (Adams and Watt, 1988) at 8th passage (kindly provided by F. Watt., ICRF, Lincoln’s Inn Fields, London) were cultured in FAD medium. HeLa cells were cultured in DME/F12 medium supplemented with 10% calf serum. Cells were subcultured by incubation with 0.1% trypsin/0.02% EDTA in PBS and were cryopreserved in liquid nitrogen as suspensions in DME/F12 medium supplemented with 10% calf serum and 10% dimethylsulfoxide.

To compare growth in different medium formulations and in the presence or absence of specific hormones, growth factors, or growth inhibitors, cells were plated at 2×10⁴ cells per 9 cm² well in 6-well plates in the desired medium, refed every 2 to 4 days, and either suspended to count or else fixed and stained with Methylene Blue 8 to 10 days after plating. When used, TGF-β1 (R&D Systems) was added to a concentration of 1 ng/ml (see Rollins et al., 1989) and phorbol myristyl acetate (PMA) (Sigma) to a concentration of 10⁻⁸ M.

Organotypic cultures were prepared as described by Parenteau et al. (1991) and by Schön and Rheinwald (1996). Briefly, 6.9×10⁴ human fibroblasts (newborn foreskin-derived, strain B038) were suspended in 3 ml of bovine collagen type I (0.7 mg/ml) (Organogenesis, Inc., Canton, MA); this suspension then was cast on top of 1 ml of an acellular collagen gel in six-well tissue culture tray inserts, the bottoms of which were polycarbonate filters of 3 μm pore size (Organogenesis, Inc.). In some experiments, myoepithelial cells...
or myoepithelial cells plus fibroblasts were embedded in the collagen gels. The cells were allowed to contract the collagen gels for 4 days at 37°C. Keratinocytes and/or myoepithelial cells then were seeded onto the gels at a density of 2×10⁶ per cm² of gel surface area and were cultured submersed for 4 days in DMEM/F12 medium (3:1 v:v) supplemented with 0.3% calf serum, 5 µg/ml insulin, 0.4 µg/ml hydrocortisone, 2×10⁻¹¹ M triiodothyronine, 5 µg/ml transferrin, 10⁻⁴ M ethanolamine, 10⁻⁴ M phosophoethanolamine, 5.3×10⁻⁹ M selenious acid, and 1.8×10⁻⁴ M adenine. The cultures were then raised to the air/liquid interface and cultured for 10 more days, after which they were embedded in OCT compound for cryosectioning.

Two-dimensional gel electrophoresis

Using methods described by Wu et al. (1982), subconfluent cultures were metabolically labeled with 100 mCi [³⁵S]methionine for 4 hours and the Triton/high salt-insoluble, cytoskeletal fraction was isolated. Equal amounts of labeled protein were resolved by non-equilibrium pH gradient electrophoresis (NEPHGE) in the first dimension and by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Labeled proteins were detected by autoradiography using Kodak X-OMAT film.

Antibodies

The following mouse monoclonal antibodies were used for immunoperoxidase staining and western blotting: keratin 5-specific antibody AE14 (Lynch et al., 1986) was kindly provided by T.-T. Sun. Keratin 7-specific antibody OVTL 12/30 (van Niekerk et al., 1991) was purchased from Biodesign International (Kennebunk, ME). Keratin 7-specific antibody K7.18 (Bartek et al., 1991), keratin 18-specific antibody CK2 (Debus et al., 1982), and vimentin-specific antibody V9 (Osborn et al., 1984) were purchased from Boehringer-Mannheim Corp. (Indianapolis, IN). Keratin 7-specific monoclonal antibodies RCK105 (Ramaekers et al., 1983) and CK7 (Töllé et al., 1985) were kindly provided by F. Ramaekers and M. Osborn, respectively. Keratin 7-specific antibody LDS-68 (Southgate et al., 1987), keratin 14-specific antibody CKB1 (Caselitz et al., 1986), and smooth muscle α-actin-specific antibody 1A4 (Skalli et al., 1986) were purchased from Sigma (St Louis, MO). Keratin 17-specific antibody E3 (Troyanovsky et al., 1989) was kindly provided by S. M. Troyanovsky. Keratin 19-specific antibody K19.1 (AS3-B/A2) (Karsten et al., 1985) was purchased from ICN Biomedicals, Inc. E-cadherin-specific antibody E4.6 (Cepek et al., 1994) was kindly provided by M. B. Brenner.

Immunocytological and immunohistochemical staining

Cultures grown in plastic tissue culture plates or multiwell trays were rinsed briefly in water and fixed for 15 minutes to 4 weeks in cold (20°C) methanol. They then were air-dried for 15 minutes and fixed for 15 minutes to 4 weeks in cold (20°C) methanol. They then were fixed with 10% formalin in PBS. The cultures were then incubated in the air/liquid interface and cultured for 10 more days, after which they were embedded in OCT compound for cryosectioning.

Fluorescence-activated cell scanning (FACS)

Cells were detached from culture dishes using 0.1% trypsin/0.02% EDTA in PBS, fixed with 1% paraformaldehyde in PBS, permeabilized with 0.3% saponin (Sigma), and rinsed in PBS. The cells then were incubated in suspension with saturating amounts of primary antibody or with isotype-matched, non-immune mouse IgG, followed by FITC-conjugated goat anti-mouse IgG. Finally, cells were rinsed twice in PBS and analyzed in a FACSscan apparatus (Becton-Dickinson) using Cell Quest Software (BD Immunocytometry Systems).

RESULTS

Isolation of morphologically distinctive epithelial cell lines from some human skin-derived keratinocyte cultures

Rare colonies of morphology different from that of typical epidermal keratinocyte colonies (Fig. 1a,c,d) were apparent in some early passage cultures initiated from cells disaggregated by trypsin from full-thickness human skin biopsies and plated in the 3T3 feeder layer system (Rheinwald and Green, 1975; Rheinwald, 1989). These ‘variant’ colonies were tightly packed but unstratified and intercellular spaces appeared wider and more refractile when viewed with phase contrast optics. Immunocytocchemical staining disclosed that, unlike keratinocytes, the variant cells did not express E-cadherin (Fig. 1b) or P-cadherin (data not shown).

Pure populations of this morphologically variant cell type were isolated from early passage mixed (predominantly keratinocyte) skin cell cultures from three different donors. In one case, CYHI, keratinocytes became nonproliferative by the end of the third passage, much earlier than usual, thereby permitting the variant cells to prevail and take over the population. From two other donors, BRSO and DOLA, colonies of variant morphology were identified in third passage cultures, isolated with cloning cylinders, and passaged subsequently as pure populations. The three variant cell lines, named CYHI, BRSO, and DOLA for the initials of their donors, were indistinguishable from one another morphologically. Their distinctive, non-keratinocyte colony morphology (Fig. 1c) remained constant during serial passage with 3T3 fibroblast feeder cells in FAD medium until they senesced after 45-60 population doublings.

BRSO, DOLA, and CYHI cells, unlike epidermal keratinocytes, grew just as rapidly (T₅₀-1 day) in FAD medium without fibroblast feeder cells, even from very low density platings (e.g. <100 cells/cm²). Under these conditions, however, the cells adopted a very different morphology, quickly losing contact with neighboring cells in growing colonies and becoming spindly or stellate (Fig. 1e), a ‘fibroblastoid’ morphology which, nevertheless, was easily
distinguishable from that of the longer and more slender human dermal fibroblast. Karyotype analysis of BRSO and CYHI disclosed that they were diploid, consistent with the conclusion that they are a normal constituent cell of skin and not an abnormal keratinocyte variant that had arisen during growth in culture.

The pattern of cytoskeletal protein expression by the ‘variant’ cell type in culture is consistent with their identity as myoepithelial cells

Seeking to identify this novel skin cell type, we used 2-D gel electrophoresis to analyze the intermediate filament proteins the cells express in culture (Fig. 2a-c). DOLA (and BRSO, not shown) expressed keratins K5, K6 (minor), K7, K14, and K16 or K17 (the latter two keratins are not resolved in this system) as well as high levels of vimentin. Western blot analysis with keratin subunit-specific monoclonal antibodies confirmed the expression by DOLA and BRSO cells of K5, K7, K17, and vimentin (Fig. 3d) and also of K14 (data not shown). The expression of K7 and high levels of vimentin by DOLA cells (Fig. 2a) distinguished these cells from epidermal keratinocytes (Fig. 2b). The expression by DOLA cells of K7 in the absence of any other simple epithelial keratin distinguished them from mesothelial cells (Fig. 2c) as well as from all other cell types characterized to date (see Wu et al., 1982; Moll et al., 1982). Indeed, coexpression of K7 and K17 is very unusual, K19 being the apparent partner for K7 in keratin filament assembly in all normal epithelial cell types studied to date (Moll et al., 1982).

When near-confluent DOLA or BRSO cultures growing in the absence of 3T3 feeder cells were refed with medium lacking EGF, the cells soon stopped dividing and adopted a more flattened morphology, some developing prominent cytoplasmic stress fibers. Many of these flat, growth-arrested cells were found to express smooth muscle α-actin (Figs 1f, 2f), reminiscent of the expression of this actin isoform by growth-arrested myoepithelial cells cultured from mammary gland (Peterson and van Deurs, 1988; O’Hare et al., 1991). These authors had also reported that mammary gland myoepithelial cells are dependent on EGF for growth in culture, consistent with the possibility that the cells we had cultured from skin were myoepithelial cells, which are a
constituent of the secretory coils of eccrine and apocrine sweat glands (reviewed by Sato, 1993; Holbrook and Wolff, 1993).

Masking of some K7 epitopes in myoepithelial cell keratin filaments, correlated with K7/K17 coexpression

Arguing against a myoepithelial identity, however, were the results of a comprehensive immunohistochemical analysis of keratins expressed during human sweat gland development (Moll and Moll, 1992), which had concluded that secretory coil cells express K7, but not K17, and that myoepithelial cells express K17, but not K7. That study employed the K7-specific antibody OVT112/30. (b and d) Immunostained with K7-specific antibody Ks 7.18. (e) Western blot of LP-9 (lane 1) and DOLA (lane 2) protein extracts, showing that the Ks 7.18 antibody recognizes K7 in the SDS-PAGE fractionated extracts of both cell lines. (Asterisk in e indicates a lower molecular mass polypeptide recognized by the antibody in the LP-9 extract, which may be a K7 proteolytic degradation product.) Bar, 200 μm, for a-d.

Fig. 3. Keratin K7 in the filaments of intact DOLA cells is not recognized by some K7-specific antibodies that recognize this keratin in the filaments of mesothelial cells. (a,b) DOLA cells; (c,d) LP-9 mesothelial cells. (a) Cultured bovine aortic smooth muscle cells; lane 2: EGF-deprived culture of BRSO; lane 3: EGF-deprived culture of DOLA; lane 4: epidermal keratinocyte strain N. For each antibody the relative electrophoretic mobility of the main immunostained band, compared with co-electrophoresed molecular mass standards, was as expected for the respective protein.

Fig. 2. Distinctive pattern of structural proteins expressed by the novel skin-derived cell type. (a-c) Cytoskeletal extracts from DOLA cells (a), epidermal keratinocyte strain N (b), and mesothelial cell strain LP-9 (c) were analyzed by two-dimensional gel electrophoresis, with non-equilibrium pH gradient electrophoretic separation in the horizontal dimension (more basic proteins left, more acidic proteins right) followed by SDS-polyacrylamide gel electrophoretic separation in the vertical dimension (larger proteins top, smaller proteins bottom). Asterisks identify the position of keratin subunits that were not completely dissociated from their filament partners during the initial isoelectric focusing step (see Wu et al., 1982; Moll et al., 1982; Franke et al., 1983). (Keratins K16 and K17 are not resolved in this system, so the presence of K17 must be determined by immunologic methods (see d). (d-f) Western blot detection of structural proteins expressed (the protein specifically recognized by the respective antibody in each extract is shown at right). (d) Lane 1: BRSO; lane 2: DOLA; lane 3: epidermal keratinocyte strain N; lane 4: mesothelial cell strain LP-9. The antibody used to detect K7 in this experiment was RCK105. (e) Lanes 1 and 2: DOLA; lanes 3 and 4: epidermal keratinocyte strain N. The cells in lanes 1 and 3 were cultured in FAD medium with 3T3 feeder cells, and the cells in lanes 2 and 4 were cultured in Gibco ker-sfm medium. (f) Lane 1: cultured bovine aortic smooth muscle cells; lane 2: EGF-deprived culture of BRSO; lane 3: EGF-deprived culture of DOLA; lane 4: epidermal keratinocyte strain N. For each antibody the relative electrophoretic mobility of the main immunostained band, compared with co-electrophoresed molecular mass standards, was as expected for the respective protein.
Table 1. Masking of epitopes recognized by some keratin K7-specific antibodies in keratin filaments of myoepithelial cells, correlated with expression of K17 as a potential pairing partner

<table>
<thead>
<tr>
<th>Keratins expressed by this cell type:</th>
<th>Mesothelial cell</th>
<th>Myoepithelial cell</th>
<th>HeLa</th>
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</thead>
<tbody>
<tr>
<td>K5</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>K7</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>K8</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>K14</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>K17</td>
<td>−</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>K18</td>
<td>+</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>K19</td>
<td>+</td>
<td>−</td>
<td>−, ±</td>
</tr>
</tbody>
</table>

(Southgate et al., 1987), did not immunostain DOLA cells (Fig. 3), BRSO cells, or HeLa cells (data not shown). The HeLa cell line is unusual in that it expresses K7, K8, K17, K18, and only trace amounts of K19 (Moll et al., 1983). As summarized in Table 1, the results we observed are consistent with the conclusion that the epitope(s) on K7 recognized by many K7-specific monoclonal antibodies is masked in cells in which K7 pairs with K17 in filament formation instead of with its more common partner, K19.

Identification of K7+/K17+ myoepithelial cells in sweat glands of human skin in vivo

We used a K7-specific antibody, OVTL 12/30 (van Niekerk et al., 1991), that could recognize this keratin in the filaments of DOLA cells to stain cryosections of human scalp skin. As shown in Fig. 4 and summarized in Table 2, of all the cell types and structures in skin, only the myoepithelial cell component of sweat gland secretory coils exhibited the same pattern of structural protein expression as DOLA and BRSO cells in culture, namely, K5/K14, K7/K17, vimentin, α-smooth muscle actin, and an absence of E-cadherin. The cells we have cultured are therefore almost certainly normal sweat gland myoepithelial cells.

Fig. 4. The pattern of structural protein expression of eccrine sweat gland myoepithelial cells detected immunohistochemically in vivo is identical to that of DOLA and BRSO cells in culture. Cryostat sections of human scalp skin were immunostained for the following proteins: (a and c) K7, (b and d) K17, (e) K14, (f) vimentin, (g) α-smooth muscle actin, and (h) E-cadherin. (a and b) S indicates a sebaceous gland, H indicates a hair follicle, and E indicates an eccrine sweat gland. (d-f) Asterisks indicate the luminal, secretory cell layers of eccrine gland secretory coils. (d-f,g,h) Open arrowheads indicate the ductal portions of eccrine glands. (b) The arrows indicate the outer, unstained myoepithelial cell layer of the eccrine gland secretory coil. Bars: 100 μm (a and b); 50 μm (c-h).
Sweat gland myoepithelial cell differentiation

Reversible modulation of myoepithelial cell morphology and keratin expression under different conditions of culture

The epithelioid-to-fibroblastoid transition of myoepithelial cells when cultured in the absence of 3T3 feeder cells, described above, proved to be accompanied by marked changes in keratin protein expression (Fig. 5). In the ‘fibroblastoid’ state, K5 and K14 content decreased in most cells to undetectable levels, K7 increased, and K17 remained about the same. We found that this altered morphology and keratin expression pattern was rapidly reversible; when DOLA or BRSO cells that had been serially passaged for ten doublings or more in the absence of 3T3 feeder cells were then replated with feeder cells, they converted back to an epithelioid morphology within several days and re-expressed K5 and K14, while reducing their level of K7. This ‘switching’ of keratin expression was quantitated by FACScan analysis (Fig. 6). We do not yet know the mechanism by which the presence of 3T3 fibroblast feeder cells exerts this marked phenotypic change in myoepithelial cells. Preliminary experiments disclosed that co-cultivation with irradiated human dermal fibroblasts also induced conversion of morphologically fibroblastoid myoepithelial cells back to an epithelioid phenotype, but feeding morphologically fibroblastoid myoepithelial cells with medium conditioned by a confluent culture of 3T3 cells did not induce epithelioid conversion. Interestingly, DOLA cells transduced to express the HPV16 E6 and E7 oncoproteins did not arrest growth or induce expression of α-smooth muscle actin in the absence of EGF and also did not convert to epithelioid morphology when cultured with 3T3 feeder cells (data not shown).

Behavior of myoepithelial cells cultured with keratinocytes and fibroblasts in Type I collagen organotypic cultures

In light of the remarkable morphologic plasticity and modulation and switching of keratin expression patterns under different conditions of conventional culture, we attempted to study their differentiation potential in three-dimensional, ‘organotypic’ culture under conditions that would permit them

Table 2. Immunohistochemical detection of filament and junctional proteins in the epithelial structures of human skin

<table>
<thead>
<tr>
<th>Structure</th>
<th>K5</th>
<th>K7</th>
<th>K8</th>
<th>K14</th>
<th>K17</th>
<th>K19</th>
<th>Vim</th>
<th>α-s.m.</th>
<th>actin</th>
<th>E-cad</th>
</tr>
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<tbody>
<tr>
<td>Epidermis</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hair follicle</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sebaceous gland</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Eccrine duct</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Eccrine secretory</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Eccrine myoepithelium</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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</table>

Fig. 5. The feeder fibroblast-modulated morphologic transition of myoepithelial cells is accompanied by alterations in keratin expression. DOLA cells were cultured in FAD medium for eight days with (a-e) or without (f-j) 3T3 feeder cells, such that they adopted an epithelioid or fibroblastoid morphology, respectively. They were then immunostained for the indicated proteins: (a,f) keratin K5; (b,g) keratin K14; (c,h) keratin K7 (antibody OVTL 12/30); (d,i) keratin K17; (e,j) vimentin. Bar, 100 μm, for all panels.
to interact with a Type I collagen gel matrix and with other skin cell types. Myoepithelial cells were co-cultured in organotypic culture with either epidermal keratinocytes, dermal fibroblasts, or both (Fig. 7). When plated on the surface of a Type I collagen gel that contained dermal fibroblasts and cultured at the air-liquid interface, myoepithelial cells formed a single cell layer, very different from the stratified, differentiated epidermal tissue structure formed by keratinocytes (Fig. 7a,b). When embedded in a collagen gel with dermal fibroblasts, myoepithelial cells remained as single cells or formed small aggregates aligned along collagen fibers but did not form complex structures, such as ducts, discernible at the level of light microscopy. When plated as a mixed cell suspension with keratinocytes on the surface of organotypic cultures, myoepithelial cells tended to sort into clusters, preferentially aggregating with one another on the collagen layer. They also formed close cell-cell attachments with surrounding keratinocytes such that they moved upward with them to the level of the granular layer when the keratinocytes formed a stratified, keratinized epithelium (Fig. 7c). However, there was no evidence of pore formation similar to the acrosyringium formed by eccrine sweat gland duct cells as they penetrate the epidermis in vivo. Myoepithelial cells embedded alone within a collagen gel adopted a spindle shaped morphology identifiable by phase microscopy and contracted the gel, similar to the behavior of dermal fibroblasts (data not shown).

Identification of proliferative myoepithelial cells as rare constituents in cultures of other skin biopsies
We sought to identify and culture myoepithelial cells from eight additional full-thickness skin biopsies in order to estimate...
the population density of proliferative myoepithelial cells in skin and to attempt to generate additional primary lines of this cell type. Rare colonies of myoepithelial cells were identified in second and third passage cultures initiated from three of these biopsies, JAKE, BABU, and C5-SkH (see Fig. 1a-c). At the time of their first detection, colonies of myoepithelial cells were present in these three cultures at only about 1/1000-1/10,000 the frequency of keratinocyte colonies and at only about 1/10-1/100 the frequency of fibroblast colonies. We were able to confirm the identity of myoepithelial cells by immunostaining cultures enriched in these cells, generated by isolating regions of cultures containing colonies of characteristic myoepithelial morphology. However, we were unable to obtain pure myoepithelial cell cultures free from keratinocyte and/or fibroblast contamination before senescence limited their growth.

We sought to identify selective conditions for obtaining pure populations of myoepithelial cells from mixed skin cell cultures by comparing the growth rates of myoepithelial cells with those of epidermal keratinocytes and dermal fibroblasts in various medium formulations and in the presence of several keratinocyte growth inhibitors. BRSO and DOLA grew with similar doubling times in a variety of culture medium formulations, including DME/F12 and M199, supplemented with calf serum, EGF, and HC, and also in Gibco ker-sfm supplemented with EGF, HC, and either bovine pituitary extract or calf serum. Similar to keratinocytes and unlike fibroblasts, BRSO and DOLA cells were strongly inhibited by TGF-β and by PMA (data not shown). We have not yet identified conditions that are simultaneously permissive for myoepithelial cell growth and selective against both keratinocytes and fibroblasts.

The ndk cell line exhibits characteristics of myoepithelial cells

The origin and identity of the ‘ndk’ line, a human skin-derived cell line with unusual properties (Adams and Watt, 1988), has remained unresolved. Ndk was isolated from newborn foreskin as a population of morphologically distinctive cells that grew during serial passage in the 3T3 feeder layer system and possessed a longer, albeit finite, replicative lifespan than the keratinocytes that had predominated in the first several passages. The ndk cells were reported to have a slight karyotypic abnormality, to express keratins similar by electrophoretic analysis to those of epidermal keratinocytes, and to be unable to form cornified envelopes. As shown in Fig. 8, our immunocytochemical analysis disclosed that ndk cells express α-sma when deprived of EGF and they express K17 and K7, the latter recognizable in intact cells by the OVTL 12/30 but not by the Ks 7.18 antibody. These results suggest that ndk is of myoepithelial origin and is not a non-differentiating, mutational variant that arose from a normal epidermal keratinocyte of the donor. The ndk cells exhibited some differences from our BRSO and DOLA myoepithelial cell lines, perhaps as a result of their chromosome abnormality or because they were near replicative senescence when we examined them. They did not convert to an epithelioid morphology when plated with 3T3 cells, a small fraction of the cells in the population expressed K19, and a similar fraction were immunostained by the Ks 7.18 antibody. Whether the

Fig. 8. Ndk cells express structural proteins consistent with a myoepithelial origin. Ndk cells at 8th passage were cultured in multiwell plates for 8 days in FAD medium. A phase contrast view of the cells is shown in a. Cells were immunostained for the following proteins: (b) keratin K17; (c) keratin K7 (antibody OVTL 12/30); (d) keratin K7 (antibody K7.18); (e) keratin K19; (f) α-smooth muscle actin (the cells in this well had been refed with medium lacking EGF for the final five days before fixation). Bar, 200 μm, for all panels.
K7.18* cells were the same cells that were K19* in the ndk culture was not determined, but it seems likely that at least some K7 in the K19* cells paired with K19 instead of K17 to form filaments, exposing the epitope recognized by the K7.18 antibody.

**DISCUSSION**

Having begun with the objective of determining the identity of a novel cell type that occasionally appears in human epidermal keratinocyte cultures, we have identified cell type-specific markers and have determined permissive culture conditions for sweat gland myoepithelial cells. The in vitro growth requirements of skin myoepithelial cells, most notably their dependence upon EGF and HC in addition to mitogens present in serum or pituitary extract, are consistent with the results of earlier studies of human mammary gland myoepithelial cells in short-term or mixed cell cultures (Peterson and van Deurs, 1988; O'Hare et al., 1991) and are similar to those previously identified for two simple (as distinguished from stratified) epithelial cell types, mesothelial cells and kidney tubule epithelial cells (Connell and Rheinwald, 1983; Rheinwald and O'Connell, 1985).

Our study discovered the expression of cytokeratin K7 by myoepithelial cells, which had not been detected previously (Moll and Moll, 1992) using an antibody that recognizes this keratin in other cell types, including the secretory coil cells of the sweat gland. Comparing the keratin expression patterns and immunocytochemical staining characteristics of cultured myoepithelial cells, mesothelial cells, and HeLa cells led us to the conclusion that when cells express K7 and K17 but not K19, K7 pairs with K17 in filament formation and results in masking of epitopes recognized by several commonly used K7-specific monoclonal antibodies. K7/K17 coexpression is very unusual. Immunohistologic detection of K7/K17 pairing in cells, accomplished by using an appropriate set of K7 and K17 antibodies as we have reported here, could be used diagnostically to aid in identifying normal and neoplastic cells of myoepithelial origin in skin and other tissues.

The extraordinary degree of morphologic and differentiative plasticity of the myoepithelial cell in culture was unexpected, considering the limited ability of sweat glands to regenerate in developmentally mature skin. The cells responded to the presence of serum, when in the absence of fibroblast feeder cells, with a pronounced elongation to a spindle shape accompanied by an altered pattern of keratin expression. We do not know whether there is a precise analogue of this situation in vivo, although structural disruption and exposure to serum factors during the several days following deep incisional or abrasive skin wounding could elicit in myoepithelial cells some of the changes we observed in culture. The plasticity and considerable proliferative potential of myoepithelial cells is consistent with a potential role for this cell type in skin wound healing in addition to its structural function in the intact sweat gland. Myoepithelial cells may contract and remodel collagen fibers in granulation and scar tissue, a possibility supported by the fact that this cell type can contract a Type I collagen gel in culture.

The ability to switch between an epithelial and mesenchymal cell morphology and pattern of protein expression occurs as part of the morphogenesis of many tissues during development but it is a very uncommon property among somatic cell types in the adult. Mesothelial cells and renal cortical tubule epithelial cells are two other cell types that can undergo a similar epithelial-mesenchymal conversion in culture and, presumably, under certain conditions in vivo (Connell and Rheinwald, 1983; LaRocca and Rheinwald, 1984; Rheinwald and O'Connell, 1985). We found that myoepithelial cells stably transfected to express the HPV16 E6 and E7 proteins were unable to arrest growth and express α-sma when cultured in the absence of EGF and also were unable to convert to an epithelial phenotype when co-cultured with 3T3 feeder cells. This indicates that the ability to modulate expression of both of these features of myoepithelial cell differentiation requires normal p53 and/or pRB dependent cell cycle regulation. The myoepithelial cell’s ‘epithelial/mesenchymal’ switching differs from that of the mesothelial and kidney tubule cell in that the myoepithelial cell continues to express two keratins, K7 and K17, in its morphologically fibroblastoid state, whereas mesothelial cells and kidney tubule epithelial cells become completely keratin-negative.

The ndk cell line, isolated as a morphologic variant from an epidermal keratinocyte culture by Adams and Watt (1988), has proved to express distinctive myoepithelial markers, K7/K17 and α-sma, suggesting that this cell line is not a differentiation defective keratinocyte mutant, as had been proposed initially. The late passage ndk cells we examined exhibited slight differences from our three diploid primary myoepithelial cell lines, however. The ndk cells did not reacquire an epithelioid morphology when cultured with 3T3 feeder cells and a small proportion of the cells expressed K19. This may be a result of the chromosone 1 abnormality identified in this cell line (Adams and Watt, 1988).

Both the apocrine and eccrine sweat glands of human skin contain myoepithelial cells. The cells we cultured were derived from full-thickness skin specimens, most of which were from axilla, so our myoepithelial cell lines could be of either eccrine or apocrine origin. We were unable to obtain specimens of human skin from which the relatively rare apocrine glands could be identified in sections for immunohistochemical analysis. Ultrastructural examination has identified myoepithelial cells in the secretory coil of apocrine glands as well as eccrine glands (for example, see Saga and Takahashi, 1992). Although the developmental formation of these two types of sweat glands is different, they form at different times from separate cell lineages in the primitive epidermis, the function of myoepithelial cells in all glands that contain them appears to be the same. Myoepithelial cells from eccrine, apocrine, and even mammary, salivary, and lacrymal glands would, therefore, be expected to have very similar patterns of gene expression and regulation. Possible differences among them might include differential responsiveness to reproductive hormones and neurotransmitters which regulate the function of some of these glands and may affect the myoepithelial cells directly.

The stringent growth requirement of myoepithelial cells for EGF and HC, even in the presence of high concentrations of serum, is also a characteristic of human mesothelial cells and kidney tubule epithelial cells (Connell and Rheinwald, 1983; Rheinwald and O’Connell, 1985). However, in contrast to the
latter cell types, myoepithelial cells are inhibited by TGF-β and by PMA, resembling the keratinocyte in this respect. Further research on the biology of this cell type will be aided greatly by identifying a culture medium formulation that promotes myoepithelial cell proliferation while completely or substantially preventing keratinocyte and fibroblast proliferation, thereby permitting routine initiation of primary myoepithelial cell lines from full-thickness skin biopsies. Rare proliferative myoepithelial cells in skin biopsies could potentially be selected out from the large population of epidermal keratinocytes by culturing in serum-supplemented Gibco ker-sfm medium without 3T3 feeder cells. However, any dermal fibroblasts also present in such cultures can eventually outgrow the myoepithelial cells in this and all other medium formulations we have tested to date. Enriched myoepithelial cell populations have been obtained from mammary gland cell suspensions by antibody-based cell sorting, exploiting the expression of the CALLA antigen by mammary gland myoepithelial cells in vivo (O’Hare et al., 1991; Gomm et al., 1995). Although myoepithelial cells are present at several orders of magnitude lower proportion of the total cell population in skin than in the mammary gland, such a strategy may aid the isolation of pure sweat gland myoepithelial cell populations.

The repair and regeneration capacity of skin adnexal structures and the regulatory mechanisms involved are subjects of great interest, in part for the potential applicability of this knowledge to developing improved transplantation and pharmacologic strategies for restoring skin damaged by burns. The epidermis can be restored permanently on large areas of full thickness wounds by applying sheets of cultured autologous epidermal keratinocytes to residual dermal or granulation tissue (O’Connor et al., 1981; Leigh et al., 1991) but regenerating the very complex structures of the pilsaceous unit and the eccrine sweat gland presents a much greater challenge (reviewed by Martin, 1997). The patterns and density of these adnexae in the normal adult skin are determined and the structures themselves formed during midgestation development. Pluripotent progenitor cells, which in the embryonic epidermis migrate downward, replicate, and ultimately differentiate into the three cell types of the mature eccrine sweat gland, are unlikely to be present in adult skin. After a partial thickness wound, the eccrine sweat duct can reestablish its connection through the regenerated epidermis to the skin surface. Duct cells, therefore, must retain both replicative potential and the ability to form their appropriate three dimensional structure, presumably using the deeper, undamaged part of the duct both as a source of stem cells and as a template. In full thickness wounds, however, both the duct and the secretory coil of the sweat gland are destroyed. Although the surface of such a wound may become reepithelialized by keratinocytes migrating from the periphery of the wound and differentiating to reestablish the epidermis, sweat glands do not regenerate.

Cells of the sweat gland secretory coil normally undergo very little turnover and replacement (for example, see Morimoto and Saga, 1995). The type and extent of damage and disruption from which this structure can recover by repairing itself and reestablishing connection with remnants of the duct is unknown, however, and the proliferative potential of the myoepithelial cells is consistent with regeneration potential. Our initial attempt to assess the histogenic potential of myoepithelial cells in vitro, using a Type I collagen matrix organotypic culture system with dermal fibroblasts and epidermal keratinocytes, was inconclusive. Culturing myoepithelial cells in the presence of basement membrane collagens and laminins, sweat gland duct and secretory cells, and soluble morphogenetic proteins such members of the BMP family would provide a more comprehensive in vitro assessment of the histogenic potential of this cell type. The development of methods for identifying and culturing myoepithelial cells which we have reported here, combined with methods for initiating cultures of eccrine duct and secretory cells (Collie et al., 1985; Lee et al., 1986; Jones et al., 1988; Pedersen, 1989) provides a starting point for investigations aimed at understanding the regenerative potential of the sweat gland and at transplanting cultured cells to restore these structures in damaged skin.

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