The 50- and 58-kdalton Keratin Classes as Molecular Markers for Stratified Squamous Epithelia: Cell Culture Studies

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ABSTRACT The keratins are a highly heterogeneous group of proteins that form intermediate filaments in a wide variety of epithelial cells. These proteins can be divided into at least seven major classes according to their molecular weight and their immunological reactivity with monoclonal antibodies. Tissue-distribution studies have revealed a correlation between the expression of specific keratin classes and different morphological features of in vivo epithelial differentiation (simple vs. stratified; keratinized vs. nonkeratinized). Specifically, a 50,000- and a 58,000-dalton keratin class were found in all stratified epithelia but not in simple epithelia, and a 56,500- and a 65–67,000-dalton keratin class were found only in keratinized epidermis. To determine whether these keratin classes can serve as markers for identifying epithelial cells in culture, we analyzed cytoskeletal proteins from various cultured human cells by the immunoblot technique using AE1 and AE3 monoclonal antikeratin antibodies. The 56,500- and 65–67,000-dalton keratins were not expressed in any cultured epithelial cells examined so far, reflecting the fact that none of them underwent morphological keratinization. The 50,000- and 58,000-dalton keratin classes were detected in all cultured cells that originated from stratified squamous epithelia, but not in cells that originated from simple epithelia. Furthermore, human epidermal cells growing as a monolayer in low calcium medium continued to express the 50,000- and 58,000-dalton keratin classes. These findings suggest that the 50,000- and 58,000-dalton keratin classes may be regarded as "permanent" markers for stratified squamous epithelial cells (keratinocytes), and that the expression of these keratin markers does not depend on the process of cellular stratification. The selective expression of the 50,000- and 58,000-dalton keratin classes, which are synthesized in large quantities on a per cell basis, may explain the high keratin content of cultured keratinocytes.

The keratins are a family of water-insoluble proteins that form tonofilaments (a class of intermediate-sized filament), which are present in almost all vertebrate epithelia (13, 16, 17, 59, 61, 62). Since keratins are usually not detectable in nonepithelial tissues including fibroblasts, muscles, and nerves, immunohistochemical staining of keratins facilitates the detection and identification of epithelial cells, both in tissue section and in culture (1, 2, 7, 8, 24, 35, 37, 46–48, 54, 55, 69).

Biochemical data indicate that the polypeptide composition of keratin filaments varies depending on epithelial cell type (12, 14, 15, 18, 21, 26, 39), cellular growth environment (3, 12, 20, 22, 23, 32, 53, 60), histological differentiation stage (6, 9, 21, 49, 51, 56, 66, 67, 70), and embryonic development period (4, 11). It is therefore not surprising that many keratin species have been described in the literature. To study the biological significance of keratin heterogeneity, we have previously prepared three monoclonal antikeratin antibodies (designated AE1, AE2, and AE3). Immunofluorescence staining data showed that these antibodies are highly specific for keratin-type intermediate filaments (64, 70). Immunoblot analysis further established that each of these antibodies recognizes a specific subset of keratin polypeptides (64, 70).

Using these antibodies, we have recently shown that mammalian epithelial keratins can be divided into at least seven major classes (40,000-, 45,000-, 50,000-, 52,000-, 56,500-,[1]

[1] This class was previously described as 56,000-dalton keratin (64, 70).

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58,000-, and 65–67,000-daltons), as defined by their immunological reactivity and molecular weight (57, 64). Tissue-distribution studies showed that the expression of specific keratin classes can be correlated with different types of epithelial differentiation. Specifically, the 40,000-, 45,000-, and 52,000-dalton keratin classes were found in almost all epithelia; the 50,000- and 58,000-dalton keratin classes were detected in all stratified epithelia, but not in any simple epithelia, whereas the 56,500- and 65–67,000-dalton keratins were unique to the keratinized epidermis (57, 64). Furthermore, immunolocalization experiments showed that, within the epidermis (which expresses the 50,000-, 56,500-, 58,000-, and 65–67,000-dalton keratin classes), the 50,000- and 58,000-dalton keratins are synthesized in the basal layer, whereas the 56,500- and 65–67,000-dalton keratins are characteristic of only cells above the basal layer (70; compare 49). Taken together, these results strongly suggest that the 50,000- and 58,000-dalton keratins may be regarded as markers for stratified epithelia, whereas the 56,500- and 65–67,000-dalton keratins may be regarded as markers for keratinization (56, 57, 64, 70; also see 40). An important implication of these results is that specific keratin classes may be useful as markers for distinguishing different types of epithelial differentiation (simple vs. stratified; keratinized vs. nonkeratinized). However, since most of our earlier studies were done using in vivo tissues, whether these keratin markers are useful for identifying cells in culture is uncertain.

Depending on the number of cell layers, in vivo epithelia can be divided into two broad categories: simple epithelia, which contain only a single cell layer, and stratified epithelia, which contain multiple cell layers. The degree of cellular stratification is not a reliable criterion for classifying cultured epithelial cells, however, since cells could undergo major morphological and structural changes in response to in vitro growth environments. For example, epidermal cells cultured in media containing a low concentration of calcium grow as a monolayer that resembles to some extent a simple epithelium (28, 29). On the other hand, cells derived from in vivo simple epithelia could "pile up" under certain culture conditions and may thus appear stratified. It is therefore clear that classification of cultured epithelial cells based solely on their morphology or histology is inadequate and sometimes even misleading.

In the present study, we analyzed keratins synthesized by a number of cultured human epithelial cell types. We found that all stratified squamous epithelia examined so far expressed the 50,000- and 58,000-dalton keratin classes, whereas cultured simple epithelia did not. Furthermore, we demonstrated that the 50,000- and 58,000-dalton keratin classes were expressed by stratified epithelial cells even when the cells formed a monolayer. Such results suggest that the expression of the 50,000- and 58,000-dalton keratin classes does not depend on the process of cellular stratification, and that these two keratin classes may represent "permanent" markers for stratified squamous epithelia, both in vivo and in vitro. Finally, we showed that the 56,500- and 65–67,000-dalton keratins were not detected in any cultured epithelial cells, reflecting the fact that none of the cells underwent phenotypic keratinization under our culture conditions.

MATERIALS AND METHODS

Monoclonal Antibodies: Mouse monoclonal antibodies AE1 and AE3 (AE is an abbreviation for antiepithelium) were prepared against SDS-denatured human epidermal (callus) keratins using the hybridoma technique (31). The preparation and characterization of these antibodies have been described in detail elsewhere (64, 70).

Cell Culture and Immunofluorescence Staining: All the cell types studied were of human origin. Human epidermal cells (44), corneal epithelial cells (59), and squamous cell carcinoma cells (SCC-12; courtesy of James G. Rheinwald of Harvard Medical School, Boston, MA; [72]) were grown in the presence of lethally irradiated mouse 3T3 cells in Dulbecco's modified Eagle's medium (DME) supplemented with 20% fetal calf serum, epidermal growth factor ([EGF] 10 ng/ml), and hydrocortisone (0.4 μg/ml; [44]). All other established human cell lines (see Figs. 1 and 2, and Results) were grown in DME containing 10% calf serum. An extract of human mesothelial cell cultures was kindly provided by Dr. James G. Rheinwald (71), and several established cell lines were generously provided by Dr. Lan Bo Chen of Harvard Medical School (Boston, MA).

Human epidermal cells were also grown under a condition that prevents cellular stratification. Epidermal cells were cultured in regular medium as described above until colonies reached a size of 20–100 cells. The cells were then fed with a "low calcium" medium (calcium-free DME containing 20% untreated fetal calf serum supplemented with 1 mM EGTA; [10, 28, 29]). Preliminary experiments established that under these conditions human epidermal cells grew to confluency as a monolayer with no detectable cellular stratification. Cultured cells grown on glass coverslips were fixed with cold methanol and stained with the monoclonal antibodies using the indirect immunofluorescence technique (61).

Keratin Extraction: Cultured cells were rinsed twice with phosphate-buffered saline (PBS), scraped from culture dishes with a rubber policeman, and homogenized in a solution containing 25 mM Tris HCl (pH 7.4) to remove the "water-soluble" proteins (60). To minimize proteolytic degradation, all extraction soltuions contained a mixture of antipain (Sigma Chemical Co., St. Louis, MO; 5 μg/ml), pepstatin A (Sigma Chemical Co.; 5 μg/ml), 1 mM EDTA, 1 mM EGTA, and 1 mM phenylmethylsulfonyl fluoride (64). After centrifugation at 10,000 *g* for 10 min at 4°C, the "water-insoluble" fraction was dissolved by heating at 95°C for 10 min in 1% SDS containing 25 mM Tris HCl (pH 7.4) and 10 mM dithiothreitol (DTT). Total proteins were prepared by dissolving the cells directly in 1% SDS with 25 mM Tris HCl (pH 7.4) and 10 mM DTT followed by heating at 95°C for 10 min. Water-insoluble and total proteins were cleared of SDS-insoluble material and other debris by centrifugation for 5 min in a microcentrifuge (model 235 A, Fisher Scientific Co., Allied Corp., Pittsburgh, PA). Water-insoluble and total proteins of in vivo human abdominal and breast epidermis were isolated using a similar procedure (70).

Gel Electrophoresis and Immunoblot Analysis: Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS PAGE, 12.5% acrylamide; [33, 60]) and then electrophoretically transferred to nitrocellulose paper (Millipore filters, type HA with a 0.45 μm pore size, Millipore Corp., Bedford, MA) using an E-C blotting apparatus (2.5 h at 4°C with a power supply setting of 65%; [63, 70]). These blots were then stained with the mouse monoclonal antikeratin antibodies by the peroxidase-antiperoxidase (PAP) technique (70; compare 27, 64).

RESULTS

50,000- and 58,000-dalton Keratin Classes Are Characteristic of Cultured Stratified Squamous Epithelial Cells

To characterize the keratin-related proteins expressed by cultured epithelial cells, we prepared water-insoluble cytoskeletal proteins of various cultured human cell types and subjected them to immunoblot analysis with AE1 and AE3 monoclonal antikeratin antibodies. As we have shown earlier, these two antibodies recognize most, if not all, known keratins expressed by various in vivo epithelial tissues (57, 64).

AE1 Antibody

Previous immunoblot experiments showed that in the in vivo epidermis, AE1 antibody reacts with a 50,000-dalton keratin class common to all stratified squamous epithelia, as well as a 56,500-dalton "epidermis-specific" keratin (Fig. 1, lane 1; [70]). Immunoblot analysis of water-insoluble cytoskeletal proteins (30 μg) extracted from various cultured cell

types demonstrated the presence of the 50,000-dalton keratin class (consisting of a 50,000- and a 48,000-dalton component as detected by the AE1 antibody; see Discussion) in all stratified squamous epithelial cells, including both primary cultures and established lines. These include human epidermal cells (Fig. 1, lane 2), corneal epithelial cells (Fig. 1, lane 3; [12, 59]), an established squamous cell carcinoma line (SCC-12; Fig. 1, lane 4), an epidermoid carcinoma cell line (ME-180; Fig. 1, lane 5), and an epidermoid cervical carcinoma cell line (CRL-1550; Fig. 1, lane 6; [42]). In contrast, cultured cells derived from in vivo simple epithelia including mesothelial cells (Fig. 1, lane 7; [71]), a pancreatic carcinoma cell line (CRL-1420; Fig. 1, lane 8; [73]), a mammary carcinoma cell line (MCF-7; Fig. 1, lane 9; [50]), and a cervical adenocarcinoma cell line (HeLa; Fig. 1, lane 10; [25]), contained either little or no detectable amounts of the 50,000-dalton keratin class proteins.

AE1 antibody detected several other keratins in various cultured epithelial cell types. A 40,000-dalton keratin was detected in all cultured epithelial cell types examined including normal human epidermal cells (Fig. 1). This 40,000-dalton keratin has been found in a wide variety of epithelial cell types, both in vivo and in culture (15, 22, 34, 40, 64), but is undetectable in normal in vivo epidermis, even using the highly sensitive immunoblotting technique (Fig. 1; [70]). However, this keratin is expressed in embryonic epidermis (4), and appears to be expressed at an elevated level in certain cell lines originated from oral- or skin-derived squamous cell carcinomas (72).

The 56,500-dalton keratin class, which may be regarded as a marker for keratinization (Fig. 1, lane 1; [56, 57, 64, 70]), was not detected in any of the cultured epithelial cell types (Fig. 1). This agrees with the fact that none of these cells undergo morphological keratinization (74) as judged by the absence of membrane-coating or keratohyalin granules.

Consistent with earlier immunofluorescent staining data that showed that keratin is epithelial-specific (13, 16, 61, 62), AE1 antibody did not react significantly with any proteins of the nonepithelial cell types, including a myeloma cell line (RPMI-8226; Fig. 1, lane 11; [38]), a neuroblastoma cell line (IMR-32; Fig. 1, lane 12; [65]), and a number of fibroblastic cell types (for data on WI-38 human embryonic lung fibroblasts; [70]).

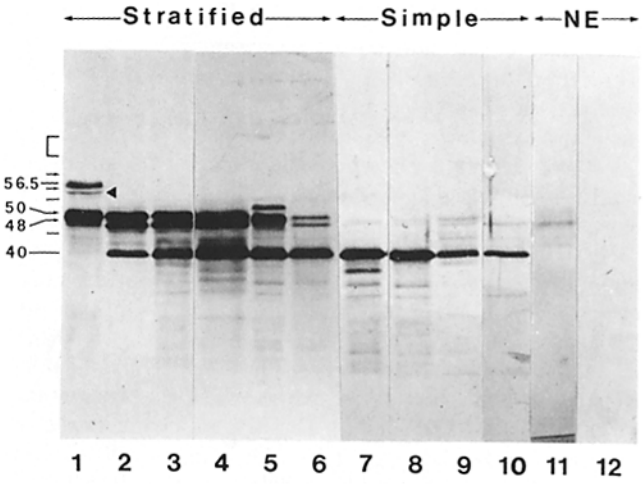


FIGURE 1 Immunoblot analysis of keratins (values at left represent the molecular weight × 10^{-3}) from various cultured human cells using AE1 monoclonal antibody. Water-insoluble proteins (30–40 μg) were isolated from various cells, subjected to electrophoresis on a 12.5% (Laemmli) SDS polyacrylamide gel, transferred electrophoretically to nitrocellulose paper, and stained with AE1 monoclonal antikeratin antibody by the PAP technique: lane *1*, keratins isolated from the living layers of human breast epidermis. (This in vivo specimen is included for comparison; all other lanes are cultured human cells.) Note the staining of a 56,500-dalton keratin (arrowhead indicates a 56,000-dalton component that was occasionally observed and presumably represented a degradative product of the 56,500-dalton keratin). Also note the absence of the 40,000-dalton keratin in normal in vivo epidermis (64, 70); lane *2*, cultured human newborn foreskin epidermal keratinocytes; lane *3*, corneal epithelial cells; lane *4*, a squamous cell carcinoma cell line (SCC-12); lane *5*, an epidermoid carcinoma cell line (ME-180): A band at 52,000-daltons with variable intensity is sometimes observed, but the significance of this band is unclear; lane *6*, a cervical epidermoid carcinoma cell line (CRL-1550); lane *7*, mesothelial cell (courtesy of Dr. James G. Rheinwald, Harvard Medical School Boston, MA): The 37,000-dalton band, which is frequently found in in vivo tissues (64), may represent a degradative product; lane *8*, a pancreatic carcinoma cell line (CRL-1420); lane *9*, a mammary carcinoma cell line (MCF-7); lane *10*, a cervical adenocarcinoma cell line (HeLa); lane *11*, a myeloma cell line (RPMI-8226); lane *12*, a neuroblastoma cell line (IMR-32). Note the presence of 50,000/48,000-dalton keratins in all cultured stratified squamous epithelial cells. *NE*, nonepithelial cells.

AE3 Antibody

As reported earlier, AE3 in normal human epidermis recognizes a 58,000- and a 65–67,000-dalton keratin (Fig. 2, lane 1; [70]). Immunoblot analysis of water-insoluble proteins extracted from various cultured cells showed that the 58,000-dalton keratin class was present in all stratified squamous epithelial cells, including primary cultures and established cell lines (Fig. 2, lanes 2–6; samples same as Fig. 1). Like the 50,000-dalton keratin class, the 58,000-dalton keratin class contained two major components (58,000 and 56,000-daltons; [64 and Discussion]). In contrast, cultured cells derived from simple epithelia (Fig. 2, lanes 7–10) contained little or no detectable amounts of the 58,000-dalton keratin class proteins.

Previous data suggest that the 65–67,000-dalton keratin class may be regarded as a keratinization marker (56, 57, 64, 70). This keratin class was readily detected in the in vivo human epidermis (Fig. 2, lane 1), but was hardly detectable in cultured epidermal cells or any other cultured epithelia (Fig. 2, lanes 2–10). However, in the same size range, a diffuse, minor band of ~66,000-daltons was detected by AE3 antibody in a wide variety of cell types including fibroblasts (Fig. 2). As we have reported earlier (64), this AE3-reactive component is also present in brain, fibroblast, and skeletal muscle, and therefore cannot be regarded as a typical keratin; it may be related to a protein recently reported to share an antigenic determinant with several types of intermediate filaments (43).

AE3 also recognized a 45,000-, a 52,000-, a 54,000-dalton keratin and several other keratin proteins in many cultured epithelial cell types (Fig. 2). Some of these keratins may correspond to the 45,000- and 52,000-dalton keratin classes previously identified in various in vivo epithelia (64). Finally, consistent with the in vivo results (64), AE3 did not react with any well-defined protein band other than the 66,000-dalton component in any of the nonepithelial cell types (Fig. 2, lanes 11 and 12).

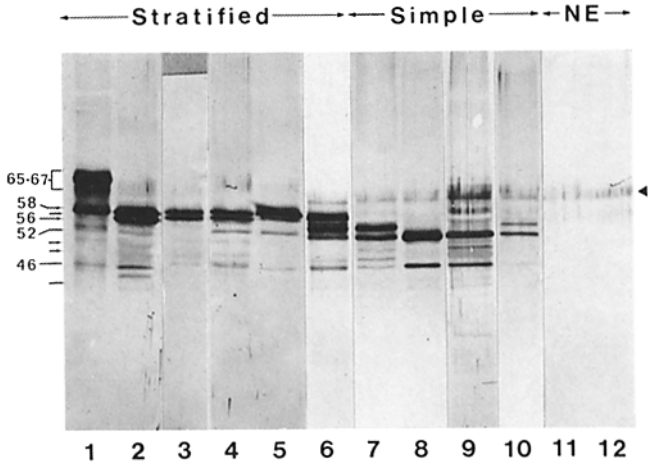


FIGURE 2 Immunoblot analysis of keratins (values at left represent the molecular weight × 10^{-3}) from various cultured human cells using AE3 monoclonal antibody. Samples were identical to those of Fig. 1. Note that the 65–67,000-dalton keratin was detected only in the in vivo epidermis (lane *1*) but not in any cultured epithelial cells (lanes *2–10*), and that the 58/56,000-dalton keratins were detected in all stratified squamous cells (lanes *2–6*). The arrowhead on the right denotes the 66,000-dalton protein previously found in preparations of various types of intermediate filaments (43, 64).

Expression of the 50,000- and 58,000-dalton Keratin Classes in the Absence of Cellular Stratification

Although under in vivo conditions a strong correlation existed between the expression of 50,000- and 58,000-dalton keratin classes, and the stratified state of the epithelia, no such correlation appeared to exist in culture. For example, mammary carcinoma cells (MCF-7) and pancreatic carcinoma cells (CRL 1420), which contained only trace amounts of the 50,000- and 58,000-dalton keratins (Figs. 1 and 2, lanes 8 and 9), could form "stratified" colonies. On the other hand, epidermoid carcinoma cells (ME-180), which contained copious amounts of the 50,000- and 58,000-dalton keratins (Figs. 1 and 2, lane 5), grew under our standard culture conditions mainly as a monolayer (data not shown).

To investigate the relationship between the expression of the 50,000- and 58,000-dalton keratin classes and the degree of cellular stratification, we grew human epidermal cells in media containing different concentrations of calcium ion. Hennings and co-workers (29) have previously shown that extracellular calcium concentration plays an important role in regulating epidermal stratification. In medium containing a high concentration of Ca^{++}, human epidermal cells stratify, with close cell-cell contacts and abundant desmosome formation (44, 58). Immunofluorescent staining of such cells using antikeratin antibodies clearly demonstrated the formation of multiple cellular layers (Fig. 3 *b*, AE1 staining; Fig. 3 *c*, AE3), as well as the presence of large, superficial squamous cells (Fig. 3 *a*; AE1). In contrast, cells cultivated in low-calcium medium grew as a monolayer, with widened intercellular space, little or no desmosome formation, and perinuclearly distributed tonofilaments (Figs. 3, *d* and *e*; [29]). Discrete keratin fibers were more difficult to visualize in cells growing in low calcium medium, presumably reflecting a decreased degree of keratin bundle formation.

Immunoblot analysis with AE1 and AE3 antibodies showed that, consistent with earlier results by Henning et al. (28, 29), the 50,000- and 58,000-dalton keratin classes were present in both stratified (Fig. 4, lanes 2 and 4) and nonstratified cultures (Fig. 4, lanes 1 and 3), indicating that human epidermal cells expressed these keratin markers regardless of their state of stratification.

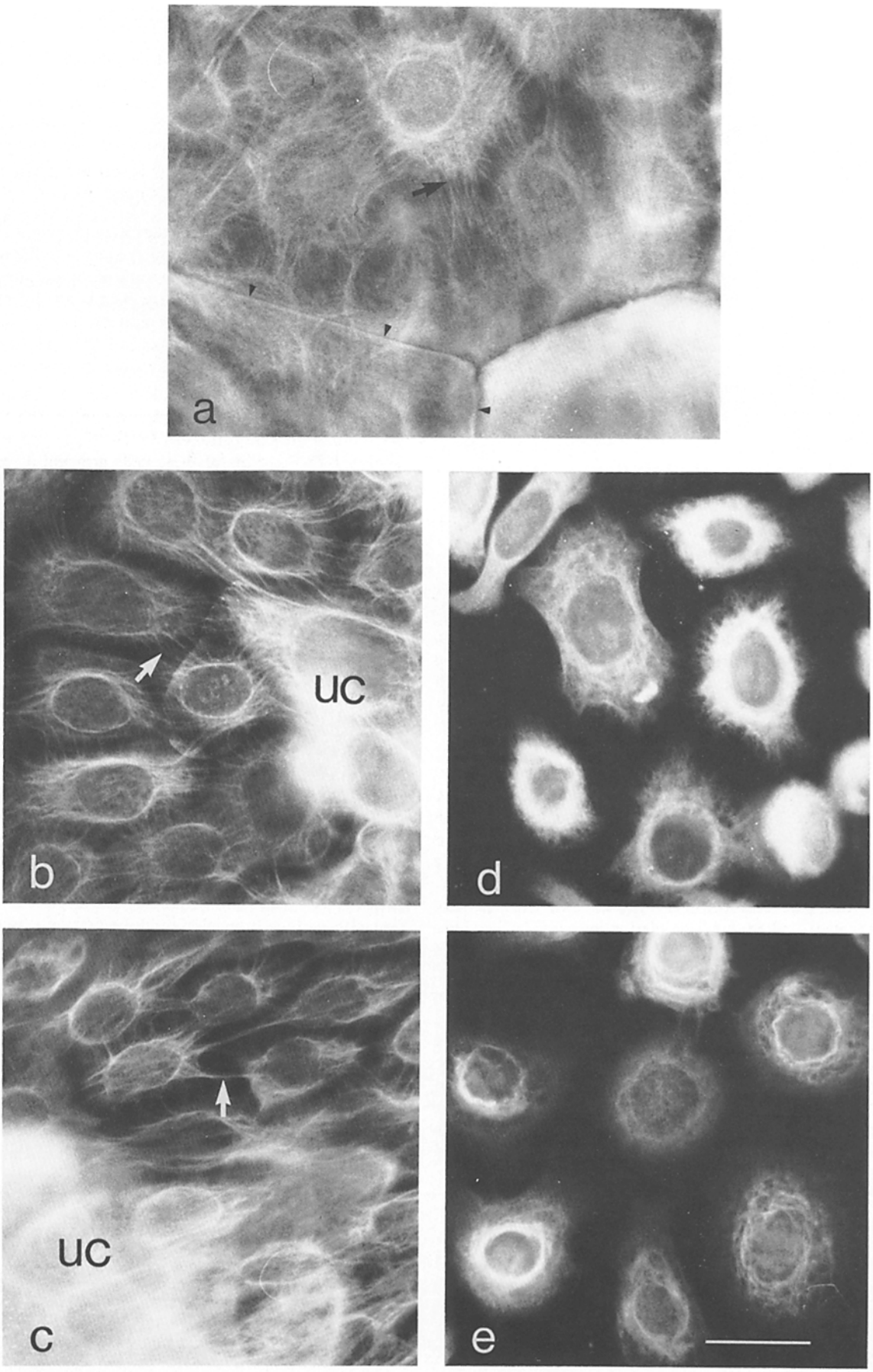
DISCUSSION

The 50,000- and 58,000-dalton Keratin Classes as "Permanent" Markers for Stratified Squamous Epithelial Cells (Keratinocytes)

Our results indicate that the 50,000- and 58,000-dalton keratin classes, as defined by AE1 and AE3 monoclonal antibody, respectively, were expressed by cultured epithelial cells of stratified squamous origin, but not by cells derived from simple epithelia (Figs. 1 and 2). These results suggest that the 50,000- and 58,000-dalton keratin classes may serve as useful markers for distinguishing the two cell types not only in vivo (57, 64), but also in cell culture.

That all stratified epithelial cells (both in vivo and in culture) so far examined expressed the 50,000- and 58,000-dalton keratins suggests that these two keratin classes may play an important structural role in such epithelia. This possibility is supported by the observation that in rabbit embryonic skin, these two keratin classes were not expressed until day 18 of gestation, when the epidermis becomes stratified (S. C. G. Tseng, J. W. Huang, and T.-T Sun, manuscript in preparation). In view of these results, it was somewhat surprising that human epidermal cells growing in a low calcium medium, although they failed to stratify, nevertheless expressed the 50,000- and 58,000-dalton keratin classes (Figs. 3 and 4; 28, 29). These observations suggest that the 50,000- and 58,000-dalton keratin classes may represent "permanent" markers that stratified squamous epithelia acquire during embryonic development, and that cells endowed with these keratin markers may gain the capability or become specialized to stratify under proper in vivo conditions. Moreover, our data also suggest that in adult epithelia the expression of such keratin markers did not depend on the process of cellular stratification.

We have previously described the definition of seven major keratin classes according to their size, immunological reactivity with various monoclonal antibodies, and tissue distribution (57, 64). According to such criteria, the 50,000- and 58,000-dalton keratin classes each contain multiple keratin species. For example, the 50,000-dalton keratin class is known to contain at least three components: a 50,000-dalton component that is characteristic of almost all stratified squamous epithelia (both in vivo and in culture), a 48,000-dalton component that is expressed mainly in hyperproliferative epidermis (69, and R. A. Weiss, R. Eichner, and T.-T. Sun, manuscript in preparation) as well as cultured keratinocytes (Fig. 1, lanes 2–6), and a 46,000-dalton component, which is a major keratin in cultured human epidermal cells (although closely related to the 50,000- and 48,000-dalton keratins, this 46,000-dalton keratin is not reactive with AE1 antibody; R. Eichner, P. Bonitz, and T.-T. Sun, (manuscript in preparation). Similarly, the 58,000-dalton keratin class contains at least two components: a 58,000-dalton component expressed by almost all stratified squamous cells both in vivo and in vitro, and a 56,000-dalton keratin component that, like the 48,000-dalton keratin described above, was expressed only in hyperproliferative epidermis and many cultured keratinocytes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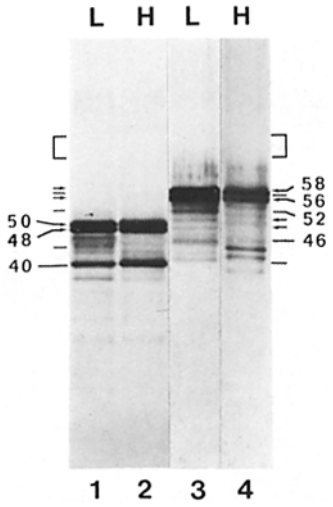

FIGURE 4 Immunoblot analyses of keratins (values at left and right represent the molecular weight × 10^{-3}) synthesized by cultured human epidermal cells growing in the presence of low (*L*) and high (*H*) calcium concentrations. Keratins were extracted from cells (Fig. 3) growing in low (lanes *1* and *3*) or high calcium medium (lanes *2* and *4*), and analyzed by the immunoblot technique using AE1 (lanes *1* and *2*) and AE3 (lanes *3* and *4*) antibodies. Approximately equal amounts (30 μg) of water-insoluble cellular proteins were applied per gel for the low and high calcium samples. Note the presence of the 50,000/48,000- and 58,000/56,000-dalton keratins in cells growing in both low and high calcium media.

(Fig. 2, lanes 2–6). These results suggest that the 50,000-dalton keratin (of the 50,000-dalton class, as defined by AE1 antibody) and the 58,000-dalton keratin (58,000-dalton class, AE3) may represent "permanent" markers for stratified squamous epithelia, whereas the 48,000-dalton keratin (50,000-dalton class, AE1) and the 56,000-dalton keratin (58,000-dalton class, AE3) may represent markers for hyperproliferative epidermis and in vitro cultured keratinocytes.

How the 50,000- and 58,000-dalton keratin classes may participate in filament formation is largely unknown. Results from in vitro reconstitution experiments suggest that at least two keratin species may be required for filament formation (36, 52). That members of the 50,000- and 58,000-dalton keratin classes may co-polymerize to form heteropolymer is suggested by the following observations. (*a*) Only two major keratins (50,000- and 58,000-daltons), one from each keratin class, are present in basal cells of normal human epidermis (49, 70). (*b*) Keratins of the two classes are distinct immunologically and biochemically (e.g., the 50,000-dalton class keratins are acidic, whereas the 58,000-dalton class keratins are neutral to basic; [40, 70]). (*c*) The expression of the two keratin classes appears to be highly coordinated (12, 20, 23, 29, 32, 59, 72). (*d*) The existence of these two keratin classes seems to be well conserved during evolution (19).

Recently, Moll et al. (40) performed a systematic analysis of keratins from various human epithelial tissues by two-dimensional gel electrophoresis. A total of 19 keratin species have so far been identified and characterized with respect to their molecular weight, isoelectric point, two-dimensional peptide mapping, and tissue-distribution. A comparison of results (64, 70) suggests that our 58,000- (of the AE3 family), 56,000- (AE3), 50,000- (AE1), and 48,000-dalton (AE1) keratins probably correspond to their keratins no. 5 (mol wt, 58,000; PI, 7.4), no. 6 (mol wt, 56,000; PI, 7.8), no. 14 (mol wt, 50,000; PI, 5.3) and no. 16 (mol wt, 48,000; PI, 5.1), respectively. Interestingly, Moll et al. (40) found that these four keratins are present in A431 epidermoid carcinoma of vulva (of stratified origin), but not in HeLa, Henle-407 embryonal intestinal cells, MCF-7 breast adenocarcinoma cells, or HT-29 colon adenocarcinoma cells (all of simple epithelial origin). In addition, data from Wu et al. (71) indicate that these four keratins are present in several cultured human stratified squamous epithelial cell types, but not in cultured mesothelial cells. These results are entirely consistent with ours and provide additional evidence that the 58,000- and 50,000-dalton keratin classes are characteristic of cultured stratified squamous epithelial cells.

It has been shown previously that, in vivo, both the transitional epithelium of the bladder and the pseudostratified columnar epithelium of the trachea contain the 50,000- and 58,000-dalton keratin classes (40, 64). Although we have not analyzed the keratins of these cells growing in culture, our finding that the 50,000- and 58,000-dalton keratins are "permanently" expressed in stratified cells, irrespective of their growth environment, suggests that these keratin markers may also be expressed in cultured bladder and tracheal epithelial cells (54, 71).

The keratinocyte is the predominant cell type in all stratified squamous epithelia. Terminally differentiated keratinocytes make involucrin, which is a precursor protein of cornified envelope (5, 45, 58, 68). Another important feature of keratinocytes is their high keratin content (up to 30–40% of total cellular proteins as compared with <5% in nonstratified epithelial cells; [59, 62]). The selective expression of the 50,000- and 58,000-dalton keratin classes, which are synthesized in particularly large quantities on a per cell basis, may explain the striking difference in keratin content between cultured keratinocytes and nonkeratinocytes.

56,500- and 65–67,000-dalton Keratin Classes as Markers for Phenotypic Keratinization

Our recent data suggest that the 56,500-dalton keratin (as specified by AE1 antibody, probably corresponding to keratin no. 10 of Moll et al.; [40]) and the 65–67,000-dalton keratin (as specified by AE3 antibody; keratins no. 1 and 2 of Moll et al.; [40]) represent molecular markers for morphological keratinization (56, 57, 64, 70). Although the detailed function of these two keratins is still unclear, the fact that they are made only by cells above the basal layer strongly suggests that they may play a role in relatively advanced stages of keratinization (70). In our present study, we were unable to detect these two keratin markers in any of the epithelial cell types

FIGURE 3 Immunofluorescence staining of cultured human epidermal cells growing in media containing different concentrations of calcium: (*a* and *b*) cells growing in medium containing high calcium stained with AE1 antikeratin antibody; (c) cells growing in high calcium medium stained with AE3 antibody. Note the large squamous cells that are located in the superficial layer (in *a* the outline of one squamous cell was marked by small arrowheads), the prominent cytoplasmic keratin bundles that terminate at desmosomal cell-cell junctions (arrows in *b* and c), and cellular stratification toward the center of the colony (*UC* = upper cells); (*d* and e) cells growing in low calcium medium stained with AE1 and AE3 antibodies, respectively. Note the wide intercellular space, the lack of prominent keratin bundles, and the absence of cellular stratification. All pictures are of the same magnification. Bar (e), 20 μm.

including human epidermal cells that formed nonkeratinized colonies in culture. It should be noted, however, that when cultured epidermal keratinocytes are provided with a proper permissive environment, such as subcutaneous sites in athymic mice (12), or vitamin A-deficient culture medium (22), they can be induced to keratinize and to re-express the 65–67,000- as well as 56,500-dalton keratins (12, and R. Eichner, P. Bonitz, and T.-T. Sun, unpublished results; compare 3, 22, 23). Thus it appears that the expression of the 65–67,000- and 56,500-dalton keratins can be modulated reversibly by the external growth environment and is tightly coupled with the process of phenotypic keratinization (56, 57, 64, 70).

Concluding Remarks

We have shown previously that the 50,000- and 58,000-dalton keratin classes provide a useful marker for in vivo stratified squamous epithelial cells (57, 64). Results from the present work allow us to extend this conclusion to cells grown in culture (with the possible exception of some viral-transformed epidermal keratinocytes; T. Hronis and T.-T. Sun, unpublished results). An important practical implication of this finding is that it allows us to establish the in vivo origin of certain epithelial cell lines. For example, HeLa cells were originally thought to be derived from a cervical epidermoid carcinoma (of stratified squamous or keratinocyte origin) that, upon careful re-examination of the original specimen some 20 years later, turned out to be a cervical adenocarcinoma (simple epithelium; [25, 30]). In the present study, we found that HeLa cells did not contain the 50,000- or the 58,000-dalton keratins, suggesting that they are indeed simple epithelia and thus supporting the revised diagnosis of cervical adenocarcinoma for the original tumor. Another practical implication concerns the issue of cross-contamination of animal cells in culture. For example, based on chromosome banding and isoenzyme analysis, Nelson-Rees and co-workers (41) showed widespread contamination of established cell lines by HeLa cells. Keratin analysis using monoclonal antibodies as described here should provide additional evidence for the contamination of certain cell lines with putative stratified squamous epithelial origin (e.g., KB oral carcinoma cells) by cells of simple epithelial origin (e.g., HeLa cells; [40]), and vice versa.

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Note Added in Proof:—We have recently found that the 56,000-dalton keratin (which belongs to the 58,000-dalton keratin class of AE3 subfamily) and the 48,000-dalton keratin (which belongs to the 50,000-dalton class of AE1 subfamily) were expressed not only in various cultured keratinocytes, but also in a wide variety of hyperproliferative epidermal diseases (Weiss, R. A., and T.-T. Sun. *J. Invest. Dermatol.* 1983, 80:337a. [Abstr.]).

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