Simple Epithelial Nature of Some Simian Virus-40-transformed Human Epidermal Keratinocytes

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ABSTRACT

Previous studies have indicated that some Simian-virus-40-transformed human epidermal keratinocytes (SV40-HE) undergo significant changes in their growth and differentiated properties. To better understand the significance of these changes, we have characterized the keratins of SV40-HE cells by one- and two-dimensional immunoblot analysis using the subfamily-specific AE1 and AE3 monoclonal antikeratin antibodies. The results indicate that our SV40-HE cells have lost the M, 58,000 (No. 5), M, 56,000 (No. 6), M, 50,000 (No. 14/15), M, 48,000 (No. 16), and M, 46,000 (No. 17) keratins that are expressed by cultured normal human keratinocytes. Instead, these cells express mainly M, 52,000 (No. 8), M, 45,000 (No. 18), and M, 40,000 (No. 19) keratins, a set highly characteristic of simple epithelial cells. Furthermore, our SV40-HE cells have ceased to express involucrin, another marker for keratinocytes, and have a greatly diminished ability to undergo in vitro stratification. These results suggest that epidermal cells can sometimes lose their keratino-ocyte features as a consequence of viral transformation. This finding may have important implications regarding the mechanisms of epithelial differentiation and tumorgenesis and in the use of keratinocyte markers for tumor diagnosis.

INTRODUCTION

Recent improvements in tissue culture technique have allowed the clonal growth and serial cultivation of normal human epidermal keratinocytes (47). In the presence of 3T3 feeder cells, epidermal cells form stratified squamous colonies and undergo terminal differentiation (28, 47, 60). Biochemical analysis showed that these cells synthesize 4 major (M, 58,000, 56,000, 50,000, and 46,000) and several minor (M, 54,000, 52,000, 48,000, and 40,000) keratins (15, 24, 62). The synthesis of some of these keratins can be modulated by cellular growth environment (6, 14, 15, 24, 25, 62). However, under most standard culture conditions, the aforementioned pattern of keratin expression appears to be remarkably stable and is observed not only in long term cultured epidermal cells but also in many permanent keratino-ocyte cell lines (6, 41, 73, 74).

Results from several laboratories have indicated that human keratinocytes can be transformed by SV40, resulting in permanent cell lines (3, 12, 53, 54, 65). Some of these cell lines become feeder-independent and can proliferate even under stringent conditions that do not normally support keratinocyte growth (53, 65; compare with Ref. 3). Such cells also show altered differentiated properties, including a reduced tendency to stratify, to form cornified envelope, and to synthesize certain keratins (53, 54, 65). However, the biological significance of such findings remains unclear.

Keratins are a group of water-insoluble cytoskeletal proteins that form 10-nm tonofilaments in almost all epithelia (16, 20, 63, 64). Recent biochemical and immunological data have shown that all known human epithelial keratins can be divided into 2 subfamilies according to their charge, immunoreactivity, and cDNA sequence. Keratins of the acidic (A) subfamily have a pi of less than 6; most of them are reactive with AE1 monoclonal antibody, and they are related to "Type I" wool keratins, whereas keratins of the neutral-to-basic (B) subfamily have a pi of 6 to 8; most of them are reactive with AE3 monoclonal antibody, and they are related to "Type II" wool keratins (for cDNA work and its relation to wool keratin, seeRefs. 23, 29–31, and 55; for 2-dimensional gel and peptide mapping data, see Refs. 4, 19, 34, and 51; and, for monoclonal antibody blotting data, see Refs. 9, 15, 57, 59, and 68). Moreover, recent data on keratin expression suggest that keratins of the 2 subfamilies are closely related. Within the 2 subfamilies, members with the same size-ranks form a "pair" and follow similar rules for expression (9, 59). Thus, the largest acidic (M, 56,000) and the largest basic (M, 65,000 to 67,000) keratins form a "pair" and are expressed mainly during keratinization (15, 25, 34, 52, 58, 67, 68, 72; see Ref. 37 for exceptions); the second largest acidic (M, 55,000) and basic (M, 64,000) keratins are coreal-specific (14, 19, 34, 51); the third largest acidic (M, 54,000) and basic (M, 59,000) keratins are present mainly in internal, nonkeratinized stratified epithelia (19, 34); the fourth largest acidic (M, 50,000) and basic (M, 58,000) keratins are present in various quantities in all keratinocytes (34, 68); and the fifth largest acidic (M, 48,000) and basic (M, 56,000) keratins are expressed in hyperproliferative keratinocytes (71). The smaller components, i.e., the M, 46,000 (A)-M, 54,000 (B) and M, 45,000 (A)-M, 52,000 (B) pairs and the M, 40,000 keratins, are mainly found in simple epithelia (17, 19, 22, 34, 35, 68, 73, 74). Interestingly, within each "pair," the basic keratin is always larger than the acidic one by approximately 8,000 to 10,000 daltons (9, 59). These results strongly suggest that acidic and basic keratins, perhaps within the so-called "pairs," may play complementary roles in vivo in tonofilament assembly and/or function and that different keratin-pairs may play different functional roles during epithelial differentiation. An important implication of this concept is that specific keratin molecules can provide useful molecular markers for different types of epithelial
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differentiation (19, 34, 51, 57, 68, 73).

In this paper, we describe the detailed characterization of the keratins of SV40-transformed keratinocytes by the immunoblot technique using the subfamily-specific AE1 and AE3 monoclonal antikeratin antibodies. The results show that our SV40-transformed keratinocytes have lost all of the major keratins normally expressed by cultured keratinocytes. Instead, they express keratins that are very similar to those of HeLa and other simple epithelial cell lines. Moreover, the cells have ceased to express involucrin, another marker for keratinocytes (2, 49, 50). Control experiments utilizing T-antigen staining exclude the possibility that our SV40-HE cultures may be contaminated by HeLa or other established simple epithelial cell lines. Our data thus suggest that the differentiation program of keratinocytes, although normally remarkably stable, can undergo major alterations as a result of viral transformation. The implications of this finding on cellular differentiation, tumorigenesis, and tumor diagnosis will be discussed.

MATERIALS AND METHODS

Antibodies. The preparation and characterization of mouse monoclonal antibodies AE1 and AE3 have been described in detail elsewhere (68, 72). The mouse monoclonal anti-intermediate-filament antibody (American Type Culture Collection, Catalogue No. TIB131) was a generous gift of Dr. Rebecca Pruss, NIH, Bethesda, MD (8, 44). Rabbit anti-(human)-involucrin antibody was provided by Dr. Robert Rice, Harvard School of Public Health, Boston, MA. (49). Hamster antisera to SV40-T-antigen was a gift of Dr. Robert Carroll, New York University Medical Center, New York, NY.

Cell Culture. Secondary or tertiary cultures of normal human epidermal cells derived from newborn foreskin were used for keratin extraction. Under "standard conditions," cells were grown in the presence of mitomycin-treated (5.0 ¥g/ml; 30 min) 3T3 feeder-cells in DMEM supplemented with 20% fetal calf serum, hydrocortisone (Calbiochem; 0.5 ng/ml), and epidermal growth factor (15 ng/ml; Refs. 47 and 48). The infected keratinocyte cultures were passaged routinely at a split-ratio of 1:3 in DMEM supplemented with 10% fetal calf serum. Growth of the infected keratinocytes became independent of feeder-layer support, generally between the third and fifth serial passages. The SV40-infected cultures were then maintained and subcultured in the absence of feeder cells. Between the 10th and 15th serial passages postinfection, most of our cell lines entered a period of growth crisis. Postc ...
SV40-HE cells (Fig. 3, a, c, e, and g) contain 3 major keratins identified as M, 52,000 [subfamily B, AE3-reactive, (Fig. 3g); No. 8 of Ref. 34], M, 45,000 [subfamily A, not recognized by AE1 or AE3 (Fig. 3); No. 18], and M, 40,000 [subfamily A, AE1-reactive (Fig. 3e); No. 19]. Such a keratin composition is very similar to that of several established simple epithelial cell lines, including HeLa cells (Fig. 3, b, d, f, and h; also see Figs. 1 and 2) which contain the same M, 52,000, 45,000, and 40,000 keratins, as well as a small quantity of the M, 46,000 keratin (subfamily A, No. 17, Refs. 5, 7, 19, and 21).

Involucrin Expression. Involucrin is a M, 92,000 protein precursor of the cross-linked (cornified) envelope (27, 49, 50). Rice and Green (49) and Banks-Schlegel and Green (2) have shown previously that antibody to involucrin stains the upper cell layers of all stratified squamous epithelia, both in vivo and in culture. This finding suggests that involucrin, like the M, 50,000 and M, 58,000 keratins, may be regarded as a marker for keratinocytes (39). Fig. 4a† shows that normal HE cells, which are (SV40) T-antigen-negative (Fig. 4e), are stained by antiinvolucrin. In contrast, no involucrin staining was observed in SV40-HE cells (Fig. 4b), which are T-antigen-positive (Fig. 4b), or in HeLa cells (Fig. 4c) which are T-antigen-negative (Fig. 4c).

**DISCUSSION**

The main conclusion derived from the present work is that our SV40-transformed human epidermal cells have lost the characteristics of keratinocytes and become reminiscent of simple epithelial cells. This conclusion is based on the following observations. (a) Keratin analysis shows that SV40-HE cells have lost most of the M, 50,000 and M, 58,000 keratins that are highly characteristic of cultured keratinocytes. Moreover, the keratins of SV40-HE cells are mainly composed of M, 52,000, M, 45,000, and M, 40,000 subunits that are typically expressed by simple epithelia, both in vivo and in culture (5, 13, 17–19, 22, 34, 36, 59, 68, 73). (b) SV40-HE cells have ceased to express involucrin. Earlier results from Watt and Green (70) have shown that this protein marker is always expressed by keratinocytes, even in monolayer cultures growing in a low-calcium medium. Therefore, the loss of the involucrin marker by SV40-HE cells cannot simply be attributed to the failure of the cells to stratify (see below). The SV40-HE cells have lost the ability to stratify, even in culture medium containing high concentrations of calcium ion (≥ 1 mm; data not shown; also see Ref. 65). Our conclusion that SV40-HE cells have gained characteristics of or have "become" simple epithelial cells is in excellent agreement with an earlier report by Taylor-Papadimitriou et al. (65), who found that their SV40-HE cells can be stained by LE61, a monoclonal antibody that normally reacts only with simple epithelia (33).

There are 2 possible explanations as to how SV40 transformation of keratinocytes can lead to a cell population that resembles simple epithelia. The first is that SV40 transformation may have selected a minor subpopulation of epithelial cells that normally have the characteristics of simple epithelia. However, this possibility is incompatible with our observation that the loss of M, 50,000 and M, 58,000 keratins, which are almost always expressed coordinately in cultured keratinocytes (6, 15, 41, 61, 73, 74), disappeared in an uncoordinated fashion from our SV40-infected cells. Thus, although our postcisis cells possess no detectable M, 50,000 keratin, they still express appreciable amounts of the M, 58,000 keratin (Fig. 2, Lane 3). This suggests that, during SV40 transformation, the expression of the M, 58,000 keratin may be more stringently conserved than that of the M, 50,000 keratin. An alternative explanation for the observed SV40-induced changes would be that SV40 transformation may have altered the genomic elements of the keratinocytes in such a way that the differentiation program of the cells may have been changed to that of a simple epithelium. Since, in the early embryo, the epidermis is a simple epithelium that only later becomes stratified, it would be interesting to consider the possibility that such a change may represent a "reversal" of the developmental process (1, 38, 69).

Thus, our SV40-HE cells, like many other simple epithelial cells (both in vivo and in culture), express as their major cytoskeletal elements the M, 45,000 acidic and M, 52,000 basic keratins (M, 45,000–M, 52,000 "pair;" see "Introduction"). Franke et al. (18) have shown recently that these 2 keratins can undergo complex formation even in moderately high concentrations of urea, suggesting that the 2 subunits may interact in vivo to form tonofilaments.

We have shown, in a recent survey (Ref. 40; also see Ref. 36), that, although a great majority of neoplasms derived from stratified squamous epithelia express the M, 50,000 and M, 58,000 keratins, a few cases apparently fail to do so. A possible explanation for this phenomenon is provided by our present data showing that keratinocytes could potentially lose their M, 50,000/58,000 keratins as a consequence of SV40 and possibly some other forms of transformation. Thus, although the detection of the M, 50,000, M, 58,000, or other keratinocyte-specific keratins establishes the stratified epithelial nature of a carcinoma (40), the absence of keratinocyte-markers (or the presence of "simple-epithelial-keratins;" Refs. 10, 11, 26, 33, and 45) should not be taken as a final proof that the carcinoma is actually derived from simple epithelia.

Taylor-Papadimitriou et al. (65) have independently derived a clonal line of SV40-transformed human keratinocytes (SVK14). These cells are feeder- and anchorage-independent (65), are impaired in their ability to form squames (65), and apparently synthesize some "HeLa-type" keratins (7). These SVK14 cells are therefore quite similar to our SV40-transformed keratinocyte lines. In a separate study, Banks-Schlegel and Howley (3) infected human epidermal cells using a subgenomic fragment of SV40-DNA instead of infectious virions. Although these cells have an increased in vitro life-span, as compared with their noninfected counterparts, and are therefore probably "established," they retain many keratinocyte features, including 3T3 feeder and anchorage dependence, as well as the synthesis of keratins typical of cultured normal epidermal cells. Additional studies will be needed to determine the basis for these differences in the biological properties of the 2 systems (as established by using infectious virions versus subgenomic DNA fragments). In any case, the above mentioned data do suggest that the process of cell establishment per se cannot be solely responsible for the loss of keratinocyte markers (see Refs. 18, 46, and 74).

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Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of the cytoskeletal proteins of SV40-transformed human epidermal keratinocytes. a, fast green (FG) staining; b, anti-intermediate filament (aIF) antibody staining. Samples are: Lane 1, normal human abdominal epidermis; Lane 2, cultured normal epidermal cells (HE); Lane 3, SV40-HE cells, postcrisis; Lane 4, SV40-HE, clone M1; Lane 5, SV40-HE, clone M7; Lane 6, SV40-HE, clone M8; Lane 7, Hep-2; Lane 8, CRL-1470; and Lane 9, MCF-7 cells. Note the strong staining of vimentin and most keratins by the anti-intermediate filament antibody and the weak staining of the M, 50,000, M, 48,000, and M, 46,000 keratins (Lanes 1 and 2; Ref. 8). a and V denote actin and vimentin, respectively. M.W., molecular weight.
Fig. 2. Immunoblot analysis of the cytoskeletal proteins of SV40-HE. a, AE1 plus AE3 antikeratin antibodies; b, AE1; c, AE3. Samples are: Lane 1, normal human epidermis; Lane 2, cultured normal HE cells; Lane 3, SV40-HE, postcrisis; Lane 4, HeLa; Lane 5, CRL-1470; and Lane 6, MCF-7 cells. In c, the upper arrowhead on the right denotes a M, 66,000, AE3-reactive component which is sometimes detected even in lanes without samples; it probably represents human Stratum corneum keratin contamination (42). The lower arrowhead denotes a M, 45,000 AE3-positive band of MCF-7 cells (Lane 6); whether this is a mammary-specific keratin or a degradative product is not known. M.W., molecular weight.
Fig. 3. Two-dimensional immunoblot analysis of the keratins of SV40-HE and HeLa cells. a and b, fast green (FG) staining; c and d, anti-intermediate filament antibody (aIF) immunoblotting; e and f, AE1; g and h, AE3. Samples are SV40-HE in a, c, e, and g and HeLa in b, d, f, and h. Note that SV40-HE cells express mainly M, 40,000, M, 45,000 and M, 52,000 keratins and small quantities of higher-molecular-weight keratins, including the M, 56,000 and M, 58,000 components (which disappear totally in all 3 SV40-HE clones). Note the similar keratin composition of SV40-HE and HeLa cells. To correlate the 1- and 2-dimensional gel data, samples similar to those being analyzed by 2-dimensional gels are also run on a side lane during the second-dimensional separation (right). 1 denotes the direction of the first-dimensional nonequilibrium pH-gradient electrophoresis, and 2 denotes the direction of the second-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Acidic proteins are on the right. V and P denote vimentin and phosphoglycerate kinase, respectively (the latter was added as a reference).
Fig. 4. T-antigen and involucrin staining of SV40-HE and HeLa cells. Samples are: a and a', normal HE; b and b', SV40-HE; and c and c', HeLa cells. a, b, and c are SV40-T antigen staining, while a', b', and c' are involucrin staining. The nuclei of SV40-HE and HeLa cells are T-antigen-positive and -negative, respectively, thus excluding the possibility that our SV40-HE cells are contaminated by HeLa or other simple epithelial cells. × 600; bar, 100 μm.
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