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Expression of Keratin Genes in Mouse Epidermis and Normal and Malignantly Transformed Epidermal Cells in Culture

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Complementary DNA (cDNA) clones constructed to the 55, 59 and 67 kilodalton (K) keratins, the major keratin synthesized in newborn mouse epidermis, were used as molecular hybridization probes to examine the expression of these genes in newborn epidermis and normal and malignantly transformed epidermal cells in culture. Transcripts of these three keratin genes are abundant in newborn epidermis. However, primary cultures of epidermal cells contain very low levels of these mRNAs. The decreased expression of these keratin genes in primary cells appears to be due to factors within the culture system. Unlike primary-cell cultures, the malignantly transformed cell line Pam 212 synthesizes keratin proteins and mRNAs similar to newborn epidermis, including the 67 K keratin. However, synthesis of the 67 K keratin in Pam 212 cells is modulated by culture factors. Keratin gene expression in another Pam line, 321, differs from that of Pam 212 cells in that decreased expression of these three keratin genes occurs. These results indicate that keratin genes that are normally expressed in vivo in epidermis may be expressed in malignantly transformed epidermal cells under conditions that do not permit expression of these genes in nonmalignant primary epidermal cells.

Epidermal keratinocytes synthesize large amounts of intermediate filaments. The subunits of these filaments are keratins,
which comprise a family of at least 10 related α-helix-rich structural proteins of 40 to 70 K [1]. There are numerous reports that the pattern of keratin synthesis can change in epidermal cells during embryonic development [2] and during terminal differentiation of adult epidermis [3–6]. Changes in keratin synthesis have also been observed during experimental skin carcinogenesis [7,8], in malignant keratinocytes [8–10], and in a variety of pathologic processes in the skin [11]. These changes in keratin expression probably occur at the transcriptional level, since individual keratins are translated from different mRNAs [12,13]. However, little is known of factors that influence the expression of keratin genes.

In order to elucidate factors that regulate the expression of keratin genes, we have constructed cDNA clones corresponding to the 55, 59, and 67 K keratins, the major keratins synthesized in newborn mouse epidermis. Using these cloned cDNAs as probes for keratin gene transcripts, we were able to provide direct evidence that modulation of keratin gene expression occurs when normal keratinocytes are placed into cell culture [15]. In this report we have used the same approach to compare the expression of these genes in newborn mouse epidermis and normal cultured keratinocytes with that of malignantly transformed epidermal cells in culture.

MATERIALS AND METHODS

Preparation of Epidermis and Cultivation of Cells

The preparation of epidermis from newborn BALB/c mice and the isolation and cultivation of epidermal cells were as previously described [14].

Labeling and Extraction of Keratins from Cultured Cells

Cultures were incubated for 12 hours in methionine-free medium to which 100 μCi/ml (spec. act. −1000 Ci/mmol) [35S]methionine (New England Nuclear) was added. Cytoskeletal extracts were prepared from cultured cells as described by Fuchs and Green [15].

Isolation of RNA

The isolation of poly(A) RNA from newborn mouse epidermis and cultured epidermal cells was as previously described [13].

Analysis of Translation Products

Poly(A) RNAs were translated in a cell-free rabbit reticulocyte lysate system containing [35S]methionine (New England Nuclear). Translation products were analyzed by polyacrylamide–SDS gel electrophoresis, as described by Laemmli [16]. The gels were subjected to fluorography using EN3HANCE (New England Nuclear) and exposed to x-ray film (Kodak, XAR-5) at −70°C. Immunoprecipitation was performed as described by Stanley et al. [17].

RNA Blot Analysis

RNA was denatured with formaldehyde as described by Lehrach et al. [18]. The RNA was fractionated on a 1.1% agarose gel and transferred by blotting to nitrocellulose paper as described by Thomas [19]. The filters were prehybridized in 50% formamide (Fluka), 5 x SSC, 20 mg/mL sodium phosphate, pH 6.5, 500 μg/ml of yeast tRNA, 0.1% SDS, and 0.05% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone. The RNA blots were prehybridized for 20 hours at 42°C. Hybridization was performed at 42°C for 20 hours in the same buffer with 1 to 2 x 10^4 cpm nick-translated 32P-labeled DNA prepared as previously described [20]. The filters were washed twice with 2 x SSC–0.1% SDS for 5 minutes each at room temperature, once with 1 x SSC–0.1% SDS for 1 hour at 68°C, and twice with 0.1 x SSC–0.1% SDS for 30 minutes each at 68°C. The blots were wrapped in Saran Wrap and exposed to x-ray film (Kodak, XAR-5) using a DuPont intensifying screen (Cronex, Lightning-Plus) at −20°C.

RESULTS

A Comparison of Keratin Synthesis in Newborn Mouse Epidermis, Epidermal Cell Cultures, and Malignantly Transformed Cells

An analysis by polyacrylamide–SDS gel electrophoresis of the keratins synthesized in newborn mouse epidermis and primary cultures of mouse epidermal cells is shown in Fig. 1 for comparison. Translation products synthesized in vitro from poly(A) RNA isolated from newborn mouse epidermis are shown in lane 1. 35S-labeled cytoskeletal extract prepared from cultured epidermal cells is in lane 3. Immunoprecipitation of the translation products of epidermal poly(A) RNA with rabbit antiserum prepared against keratins present in mouse stratum corneum [21] demonstrates that keratins which are approximately 67, 59, and 55 K are the major proteins synthesized in this tissue (Fig. 1, lane 2). Keratins that are 60 and 50 K are also synthesized in newborn epidermis, but to a lesser extent. The immunoprecipitable band that is approximately 42 K may be an incomplete translation product or the product of a different keratin mRNA. Synthesis of the 67 and 59 K keratins is greatly reduced in cultured epidermal cells (Fig. 1, lane 4). The major keratins synthesized in cultured cells are 60, 55, and 50 K. Identical results were obtained when translation products of poly(A) RNA isolated from primary cultures of mouse epidermis were run in a separate gel.
nal cells were compared with cytoskeletal extracts prepared from intact cells [13].

Next, we compared the keratins synthesized in newborn mouse epidermis with those synthesized by the malignantly transformed cell line Pam 212. The Pam line arose spontaneously after prolonged culture of mouse epidermal cells and produces squamous cell carcinomas upon injection into syngeneic animals. The derivation and properties of Pam cell lines have been previously reported [22]. Pam 212 has an epithelial morphology, grows in multiple layers, and synthesizes many normal epidermal-specific antigens. To our surprise, we found that the keratins synthesized by Pam 212 cells were very similar to those synthesized in newborn epidermis (Fig. 2). As observed for epidermis in vivo, the 67, 59, 55, and 50 K keratins are the major products.

Analysis of Primary Epidermal Cell Cultures and Pam 212 Cells for Keratin Gene Transcripts

Previously, we have shown that the inability of cultured cells to synthesize the 67, 59, and 55 K keratins was due to the absence of mRNAs coding for these keratins [13]. Therefore, it was of interest to analyze the keratin mRNAs synthesized in Pam 212 cells. Poly(A) RNA was isolated from Pam 212 cells, denatured with formaldehyde, separated by agarose gel electrophoresis, and blotted onto nitrocellulose paper. Messenger RNAs coding for the 67, 59, and 55 K keratins were detected by hybridizing the blotted RNA with 32P-labeled, cloned cDNA corresponding to these keratins. Equal amounts of poly(A) RNA isolated from newborn epidermis (Fig. 3, lanes 1, 4, and 7) and cultured normal cells (Fig. 3, lanes 2, 5, and 8) were analyzed for comparison. As shown previously, mRNAs that are approximately 1.6, 2.0, and 2.4 kilobases in length and code for the 55, 59, and 67 K keratins, respectively, are very abundant in newborn epidermis. However, their concentration is greatly reduced in primary epidermal-cell culture [13]. We did not expect to find mRNAs coding for the 67 and 59 K keratins in epidermal-cell cultures because these cells synthesize very low levels of these proteins. We were surprised to find that these cells were also lacking the mRNA coding for the 55 K keratin, because one of the major keratins synthesized by epidermal cells in culture is approximately 55 K. This result suggests that the 55 K keratin synthesized in cultured epidermal cells is coded for by a gene that is very different in sequence from that coding for the 55 K keratin synthesized in epidermis in vivo which we have cloned. It also suggests that the array of keratins synthesized by epidermal cells may be more complex than previously realized by protein analysis. Pam 212 cells (Fig. 3, lanes 3, 6, and 9) contain mRNAs homologous to the 55, 59, and 67 K keratins as expected, since all three keratins were synthesized by these cells. The level of each mRNA in Pam 212 cells is less than that detected in an equivalent amount of

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**Figure 2.** Comparison of keratins synthesized by newborn mouse epidermis and Pam 212 cells. [35S]methionine-labeled proteins were immunoprecipitated with keratin antiserum, separated by electrophoresis, and visualized by fluorography. Lane 1 contains in vitro translation products of newborn mouse epidermis poly(A) RNA. Lane 2 contains a cytoskeletal extract of Pam 212 cells.

**Figure 3.** Detection of keratin mRNAs in Pam 212 cells. One micogram of poly(A) RNA isolated from newborn mouse epidermis, primary cultures of mouse epidermal cells, or Pam 212 cells was denatured with formaldehyde, separated by electrophoresis in 1.1% agarose gel, and blotted onto nitrocellulose paper. Individual sections of paper containing the three different RNA preparations were hybridized to 32P-labeled cDNA inserts corresponding to the 55 K (lanes 1–3), 59 K (lanes 4–6), and 67 K (lanes 7–9) keratins and autoradiographed. The length of keratin mRNAs (kilobases) is indicated. Cross-hybridization was observed between the 55 K keratin mRNA and the 59 K keratin cDNA (lane 4) and the 67 K keratin cDNA (lane 7).
poly(A) RNA isolated from newborn epidermis. Pam 212 cells may also express some of the keratins synthesized in primary cultures, such as the 50 and 60 K keratins, but this question can only be answered conclusively when cDNA clones corresponding to the keratins expressed in each cell culture have been constructed.

**Differential Expression of the 67 K Keratin in Pam 212 Cells**

Synthesis of the 67 K keratin is modulated in the Pam 212 line. In several experiments, the 67 K keratin was not present in labeled extracts and the mRNA complementary to the 67 K cDNA was absent in RNA preparations. A particularly striking instance is presented in Fig. 4. Immunoprecipitates of [35S] methionine-labeled extracts of two different passages of Pam 212 were studied at different times. The 67 K keratin was present in passage 17 after 8 days in culture (Fig. 4, line 1). In the next passage, after 6 days, the 67 K keratin was not evident (Fig. 4, line 2). After 6 more days, in a sister culture it reappeared (Fig. 4, line 3). This phenomenon does not seem to be due to time after subculture, since we have demonstrated the 67 K keratin as early as 2 days after passage in other experiments. We are currently analyzing the culture factors that may modulate the expression of the 67 K keratin.

**Analysis of Keratins Synthesized in Pam 321 Cells**

We have also analyzed the keratins synthesized by another Pam line, 321. This line also arose spontaneously after long-term culture in a separate experiment and has a different morphology from Pam 212; the cells are smaller and grow in cohesive monolayer clusters (Fig. 5). When postconfluent, an upper layer forms with parallel ridges of keratinized material. Unlike Pam 212 cells, Pam 321 cells exhibit a keratin pattern similar to that observed for cultured primary epidermal cells in that keratins that are 60, 55, and 50 K are the major products (Fig. 6). Extracts as well as immunoprecipitates show that these cells also synthesize a 59 K keratin, and unlike cultured cells, they also synthesize keratins that are 47, 45, and 44 K. Eleven other Pam lines analyzed in this way failed to show expression

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**Fig 4.** Differential expression of the 67 K keratin in Pam 212 cells. Cytoskeletal extracts were prepared from different cultures of Pam 212 cells that were labeled with [35S]methionine for 12 hours. The labeled proteins were immunoprecipitated with keratin antiserum separated by electrophoresis and visualized by fluorography. Lane 1 contains an extract of passage 17 of Pam 212 after 8 days in culture. Lane 2 contains an extract of passage 18 of Pam 212 after 6 days in culture. Lane 3 contains an extract of passage 18 of Pam 212 after 12 days in culture.

**Fig 5.** Pam cell lines (phase contrast; x75). Pam 212 subconfluent (A) and confluent (B). Pam 321 subconfluent (C) and confluent (D). Notice that Pam 212 cells grow in a less coherent cluster at low density than Pam 321 and that at confluence Pam 321 cells are smaller than Pam 212 cells.

**Fig 6.** Comparison of keratins synthesized by newborn mouse epidermis and Pam 321 cells. [35S]methionine-labeled proteins were separated electrophoretically and visualized by fluorography. Lane 1 contains in vitro translation products of poly(A) RNA isolated from newborn mouse epidermis. Lane 2 contains translation products in lane 1 immunoprecipitated with keratin antiserum. Lane 3 contains a cytoskeletal extract of Pam 321 cells. Lane 4 contains Pam 321 extract in lane 3 immunoprecipitated with keratin antiserum. The positions of the 50 and 55 K bands are distorted by unlabeled IgG in the immunoprecipitate. T is an mRNA-independent artifact of the translation system.
of the 67 K keratin and had variable expression of the other keratins.

Analysis of Pam 321 Cells for Keratin Gene Transcripts

Poly(A) RNA isolated from Pam 321 cells was analyzed by Northern blotting along with RNA from Pam 212 cells for transcripts for the 67, 59, and 55 K keratins (Fig. 7). With the exception of the low levels of mRNA complementary to the 55 K cDNA, Pam 321 cells did not contain transcripts of these keratin genes and again resembled cultured primary epidermal cells. It should be noted that the level of synthesis of the 59 K keratin in Pam 321 cells is higher than that observed in primary cultures and would appear to be sufficient for its mRNA to be detected in the blotting assay. This negative result suggests that the 59 K keratins synthesized in Pam 321 and newborn epidermis are coded for by different genes. There is a slight difference in the gel migration of the 59 K keratin proteins, which is consistent with multiple forms of the 59 K keratin. Careful examination of the bands in Fig. 6 shows that the 59 K keratin synthesized in Pam 321 and synthesized to a lesser extent in primary-cell culture (Fig. 1) migrates with a slightly slower mobility than the 59 K keratin synthesized in newborn epidermis. These results again suggest that normal or malignant epidermal cells are capable of synthesizing a complex array of keratins.

DISCUSSION

In our previous report [13] and in this paper we have shown that the pattern of keratin synthesis observed in intact newborn mouse epidermis changes dramatically when epidermal cells prepared from this tissue are grown in culture. The major keratins synthesized in epidermis, of 55, 59, and 67 K, are not synthesized, or are synthesized only in greatly reduced amounts, in cultured epidermal cells. The reduced synthesis of these keratins appears to occur at the transcriptional level, since the concentration of mRNAs for these keratins is also very low in cultured cells [13; this study], and we are unable to detect precursors for keratin mRNAs in nuclear RNA isolated from these cells (our unpublished results). It is also unlikely that this altered expression is due to a programmed developmental switch that may have been induced by placing the cells in culture, since we have been able to detect transcripts of these three cloned genes in both adult [13] and embryonic skin (our unpublished results). It is more likely that the changes in keratin synthesis observed in cultured epidermal cells results from modulating factors within the culture system. Fuchs and Green [15] have recently shown that vitamin A can affect the program of keratin synthesis in cultured human epidermal cells. Epidermal cells grown in culture medium containing serum depleted of vitamin A are able to synthesize the 67 K keratin. The addition of retinyl acetate to the vitamin A-depleted medium inhibits synthesis of the 67 K keratin and stimulates the synthesis of 40 and 52 K keratins. It is not known if these effects of vitamin A are direct or indirect or if they occur at the level of transcription.

When keratinocytes from intact epidermis are grown in culture, reduced synthesis occurs for the higher-molecular-weight keratins, the 65 to 67 K keratins [6, 23–25] and the 56 K keratin [25]. Cultured epidermal cells can reacquire their in vivo keratin phenotype when injected subcutaneously into nude mice [25]. Thus there is considerable evidence that the program of keratin synthesis in epidermal cells can be modulated by factors within their environment. These different programs of keratin synthesis may reflect the differentiation state of epidermal cells. In fact, Tseng et al. [26] have recently proposed that the 56 K and 65 to 67 K keratins are markers for keratinization (terminal differentiation), since these keratins are only detected in keratinized epidermis by monoclonal antibody studies. The keratins for which we have constructed cDNA clones, the 55, 59, and 67 K keratins, probably represent keratins of this class.

The synthesis of the 55, 59, and 67 K keratins in the malignantly transformed cell line Pam 212 suggests that these cells are capable of expressing a program of keratin synthesis that is normally not expressed by primary epidermal cells under our culture conditions. This indicates that Pam 212 cells are more refractory to the modulating factors affecting primary cells. Pam 212 cells are not completely resistant to these factors, however. Under certain conditions, which we have not defined, decreased synthesis of the 67 K keratin is observed. Therefore, Pam 212 cells are not blocked in a constitutive mode of expression for this particular keratin program. Wu and Rheinwald [9] have reported similar results at the protein level for several cell lines of human squamous cell carcinomas which synthesize a 40 K keratin in culture but not the 67 K keratin; the opposite result was found when these cells were transplanted in animals and grown as tumors.

The results presented for Pam 212 cells, and other results obtained for several other Pam lines but not presented in this study, suggest that expression of the 67 K keratin is not a general feature of malignantly transformed epidermal cells. In fact, the Pam 212 line was the only line examined that expressed the 67 K keratin. Since the expression of particular keratins was variable in the different cell lines, no generalization can be made concerning expression of a particular keratin program and malignant transformation. The observation that synthesis of the 67 K keratin is modulated in Pam 212 cells may enable us to identify the modulating factors acting in culture. This should provide clues to the physiologic regulators of keratin-gene expression in normal and pathologic states.

REFERENCES


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Genetically Induced Abnormalities of Epidermal Differentiation and Ultrastructure in Ichthyoses and Epidermolyses: Pathogenesis, Heterogeneity, Fetal Manifestation, and Prenatal Diagnosis

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Comparative ultrastructural investigations on the pathomorphogenesis of inherited ichthyoses and epidermolyses have shown that such heterogeneous skin disorders may serve as model systems for genetic interactions with developmental processes, such as keratinization, or functional systems, such as dermal-epidermal junctional integrity. Most interesting from the morphologic point of view are dominantly inherited skin disorders in the ichthyosis and epidermolysis bullosa groups in which primary structural defects of structural proteins have been demonstrated that seem to be under the direct control of the mutant gene. Such structural abnormalities concern keratohyalin in autosomal-dominant ichthyosis vulgaris, the tonofilament system in hystrix-like ichthyoses, and the anchoring fibrils in dominant dystrophic epidermolyses. Taking bullos congenital ichthyosiform erythroderma (epidermolytic hyperkeratosis) as a central example, we discuss the stability of such structural defects, the heterogeneity in the ultrastructural abnormalities of clinically closely similar entities (ichthyosis hystrix Curth-Macklin, congenital reticulate ichthyosiform erythroderma), and, in the latter keratinization disorder, the presence of an unusual filament system of unknown biochemical composition in the abnormal keratinocytes. Expression of mutant genes during fetal life and fetal manifestation of such abnormalities are a precondition for the prenatal diagnosis of genetic skin disorders (bullos ichthyosiform erythroderma, epidermolysis bullosa dystrophica Hallopo-Siemen, Herlitz syndrome). Finally, problems related to the differentiation of mutant keratinocytes and of amniotic fluid cells of fetuses at risk of genetic skin disorders under the in vitro conditions of primary cell cultures are briefly discussed.
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