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RETINOYLATION OF CYTOKERATINS IN NORMAL HUMAN EPIDERMAL KERATINOCYTES

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Retinoylation (retinoic acid acylation) is a covalent modification of proteins occurring in a variety of eukaryotic cell lines. In this study, we found that proteins in undifferentiated and squamous-differentiated normal human epidermal keratinocytes were retinoylated after treatment with [^{3}H]retinoic acid. The major retinoylated proteins were identified as cytokeratins based on their profile in two-dimensional gel electrophoresis and their immunoreactivity with anti-keratin monoclonal antibodies. The covalently bound [^{3}H]retinoic acid was not removed by mild hydrolysis with methanolic-KOH indicating that it is not linked to the cytokeratins by a thioester bond. The results raise the possibility that retinoylation of cytokeratins is involved in some of the effects of retinoic acid on keratinocytes.

Epidermal cells in primary culture undergo a multistep program of squamous differentiation (1-3). This *in vitro* program mirrors many of the morphologic and biochemical changes that are seen *in vivo*. Several effectors influence the differentiation state of epidermal cells. For example, Ca^{2+} is an inducer (2, 4) and RA is an inhibitor of differentiation to the squamous phenotype (2, 3, 5).

Until recently, the molecular mechanisms underlying the effects of RA on epidermal cells and other cell types was elusive. The discovery of RA nuclear receptors (6-10) has provided insight into the role of retinoids on genomic expression. These receptors are structurally similar to members of the steroid/thyroid nuclear receptor multigene family

The abbreviations used are: RA, retinoic acid; PAGE, polyacrylamide gel electrophoresis; HEPES, 4-(2-hydroxyethyl)-1-piperazinethane sulfonic acid; PBS, phosphate-buffered saline (1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 136.9 mM NaCl, pH 7.2); SDS, sodium dodecyl sulfate; NHEK, normal human epidermal keratinocytes; KGM, keratinocyte growth medium; EDTA, ethylenediaminotetraacetic acid.

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and are both positive and negative regulators of gene expression (5). It is likely that many of the effects of RA on gene expression during squamous differentiation are mediated by these nuclear receptors.

Recently, we reported evidence that a nuclear protein in HL60 cells is retinoylated *via* a thioester bond in a dose-dependent manner (11). Furthermore, the dose-response curves for RA-induced differentiation and for retinoylation are similar (11, 12). Retinoylation is not restricted to HL60 cells and occurs in other cell types (12, 13). These results showed that retinoylation is widespread and that the response to RA of different cell types may depend on the retinoylation of specific proteins. Thus, some effects of RA on cells may be independent of RA nuclear receptors.

The characterization and identification of the retinoylated proteins is one approach that could lead to a better understanding of the functional role(s) of retinoylation. In this report, we describe retinoylation in normal human epidermal keratinocytes (NHEK) and present evidence that the cytokeratins are major substrates for this modification.

MATERIALS AND METHODS

Cells - Second passage cultures of NHEK from human foreskin were obtained from Clonetics Corp. and grown in KGM serum-free keratinocyte growth medium (4) purchased from Clonetics. Cells were cultured in 60-mm plastic tissue culture dishes at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were removed from the surface with a cell scraper. Cell number was estimated using a hemacytometer and viability was estimated by trypan blue dye exclusion.

Incorporation of [^{3}H]RA and [^{35}S]methionine - NHEK from exponentially growing cultures were trypsinized, collected by centrifugation, resuspended in KGM, and plated at a concentration of about 1.3 x 10^5 cells/60-mm dish with 4 ml of KGM. Under these conditions NHEK grow exponentially and reach confluency at or about 5 days when the density is about 2 x 10^6 cells/dish (14). At confluency NHEK become committed to terminal cell division with a time-dependent expression of morphologic and biochemical markers of squamous differentiation. These markers include decreases in colony-forming efficiency and parallel increases in both cholesterol sulfate levels and transglutaminase type I activity. These changes precede the appearance of spontaneous cross-linked envelope formation, a late differentiation marker, seen 5 days after confluency (14). While RA inhibits squamous cell differentiation it does not block the entry of NHEK into terminal cell division (14). This well-characterized multi-step program of differentiation allows the study of NHEK at specific stages of differentiation. Undifferentiated cells were obtained from late exponentially-growing cultures and differentiating cells were obtained from cultures after they had reached the confluent phase of the growth curve. One day before the cells were harvested the medium was removed by aspiration and the cells were washed with KGM. KGM (2 ml) containing either 100 nM [11,12-^{3}H]RA (40-60 Ci/mmol, Du Pont-New England Nuclear) or 20 μCi/ml of [^{35}S]methionine (1097 Ci/mmol, Du Pont-

New England Nuclear) and 100 nM unlabeled RA was added and the cells were grown for 24 h. The cells were detached by scraping, harvested by low-speed centrifugation, and then washed extensively with PBS containing 600 μg of bovine serum albumin/ml.

The cytokeratins were purified from a portion of the cells from each condition according to the method described by Franke *et al.* (15). NHEK suspended in PBS were harvested by centrifugation at 14,000 x *g* for 1 min. The cells were lysed in 1 ml of 20 mM Tris-HCl, pH 7.6, containing 140 mM NaCl, 5 mM $MgCl_2$ and 0.6 M KCl and placed on ice for 30 min. The insoluble material was collected by centrifugation at 14,000 x *g* for 2 min and extracted again with the same buffer as described above containing 1% Triton X-100. After an extraction period of 30 min the insoluble material was collected by centrifugation at 14,000 x *g* for 2 min. The pellet was washed with 20 mM Tris-HCl, pH 7.6 and then lyophilized. This fraction is highly enriched in cytokeratins. All the buffers used for the cytokeratin purification contained 1 mM phenylmethylsulfonyl fluoride, 1 mM EDTA, 2.5 μg of leupeptin/ml, and 2.5 μg of aprotinin/ml. All procedures were carried out at 4°C.

The purified cytokeratins and the total cells were delipidated by the Bligh-Dyer procedure with $CHCl_3$:CH_3OH:H_2O (1:2:0.8) (16). The denatured proteins were collected by centrifugation at 10,000 x *g* for 5 min in a microcentrifuge. This extraction was repeated about 6 times until there were < 300 cpm/ml in the supernatant fraction. The protein pellets were then dried in a centrifugal vacuum device (Savant) and dissolved in the electrophoresis sample solution.

Two-dimensional PAGE - Two-dimensional SDS-PAGE was according to O'Farrell (17). First dimension isoelectric focusing gels contained 2% LKB ampholytes (pH 3.5-10). Second dimension gels were 10-20% gradient polyacrylamide (16 cm x 18 cm x 1.5 mm). Gels were fixed, stained, and prepared for fluorography as described (18).

Immunoblotting - Proteins were transferred from the two-dimensional gel to a polyvinylidene difluoride membrane (Millipore Corp.) using a Sartoblot II semi-dry electroblotter (Sartorius Filters Inc.). The reactivity of cytokeratins with monoclonal antibodies AE1 and AE3 (generously supplied by Dr. T.-T. Sun) was visualized by immunogold immunostaining using Auro Probe BL plus streptavidin (Janssen Biotech) according to the manufacturer's instructions. AE1 detects most of the type I cytokeratins and AE3 detects all the type II cytokeratins (19, 20).

RESULTS AND DISCUSSION

As shown in Fig. 1, retinoylation of proteins in both undifferentiated and differentiating NHEK was observed. The most marked difference in the retinoylation patterns between cells grown under these two conditions was an increase in the relative level of retinoylation of proteins designated *a*, *f*, *h*, and *i* in the differentiating cells. In contrast, reduced labeling by RA was seen with the Mr 55,000 protein ($p55^{RA}$) (*arrows*, Fig. 1) and the doublet at Mr 37,000 ($p37^{RA}$)(*37*, Fig. 1) in differentiating cells compared

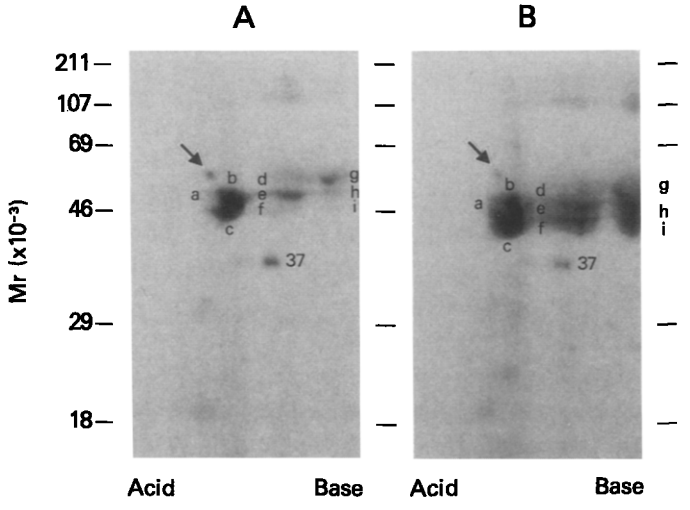

Fig 1. Two-dimensional PAGE patterns of retinoylated proteins from total cellular proteins of (A) undifferentiated and (B) differentiating NHEK. Cells from an exponentially-growing culture (undifferentiated cells) and cells from a confluent culture (differentiating cells) were labeled in KGM for 24 h in the presence of 100 nM [11,12-^{3}H]RA. Cells (6 x 10^5 undifferentiated cells and 2 x 10^6 differentiated cells) were harvested by centrifugation, washed with PBS containing bovine serum albumin, and extracted by the Bligh-Dyer procedure. The residues were dissolved in isoelectric focusing buffer (pH 3.5-10) and analyzed by two-dimensional PAGE and fluorography. The undifferentiated cell residue contained 75,000 cpm and the differentiating cell residue contained 145,000 cpm. Exposure to the film was for 48 days. ***Arrows*** and *37* show proteins that may be identical to retinoylated proteins found in HL60 cells (12). *Letters* identify proteins that have the same relative mobility in all of the figures.

to undifferentiated cells. These two NHEK proteins migrate at identical positions as [^{3}H]RA-labeled proteins identified in HL60 cells (12).

The family of cytokeratins comprise a large percentage of the total protein of keratinocytes. There are at least 21 cytokeratins involved in the formation of intermediate filaments of epidermal and other epithelial cells (21, 22). The cytokeratins are subdivided into classes type I and type II. Type I cytokeratins have Mr values of 40,000 to 56,000 and pI values of 4.9 to 5.7. Type II cytokeratins have Mr values of 52,000 to 68,000 and pI values of 6 to 7.8. The profile of the retinoylated proteins resembles that of type I and type II cytokeratins expressed in NHEK (Fig. 1).

To investigate further whether the cytokeratins are retinoylated, we prepared two-dimensional PAGE fluorograms of cytokeratin-enriched fractions and of total cell protein from differentiating NHEK grown with either [^{35}S]methionine or [^{3}H]RA. As shown in Fig. 2, *bottom*, the fluorogram of [^{3}H]RA-labeled cytokeratins was very similar to the fluorogram of [^{3}H]RA-labeled total cell proteins indicating that the cytokeratin-enriched fraction contained most of the retinoylated proteins in NHEK. The retinoylated proteins

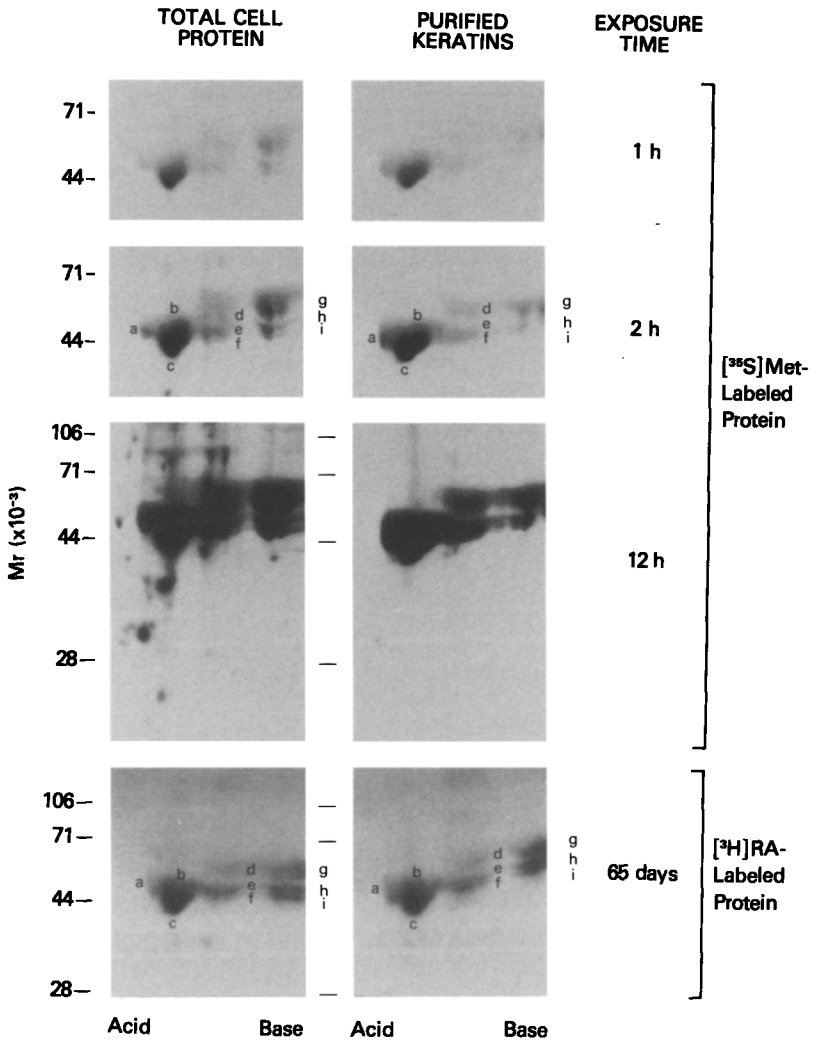

Fig. 2. **Two-dimensional PAGE patterns of total cell proteins and purified cytokeratins of NHEK labeled with either [^{35}S]methionine or [^{3}H]RA.** Cells from a confluent culture were labeled in KGM for 24 h in the presence of either 100 nM [11,12-^{3}H]-RA, 50-55 Ci/mmol or 20 μCi of [^{35}S]methionine/ml and 100 nM unlabeled RA. The cell concentrations were 2 x 10^{6}/dish. Cells were harvested by centrifugation and total cell proteins and purified cytokeratins were prepared as described under "Materials and Methods". Both preparations were extracted by the Bligh-Dyer procedure. The residues were dissolved in isoelectric focusing buffer (pH 3.5-10) and analyzed by two-dimensional PAGE and fluorography. The total cell protein residues loaded on the gel contained either 1.5 x 10^{6} cpm of ^{35}S or 18,000 cpm of ^{3}H. The purified cytokeratins residues loaded on the gel contained either 9.6 x 10^{5} cpm of ^{35}S or 9,000 cpm of ^{3}H. Exposure to the film was for 1, 2, and 12 h for the ^{35}S-labeled proteins and 65 days for the ^{3}H-labeled proteins. *Letters* identify proteins that have the same relative mobility in all of the figures. In the second-dimension gel of the retinoylated proteins from the cytokeratin-enriched fraction the relative migration of the proteins decreased from left to right across the gel. This "smiling-effect" probably was caused by a temperature variation from one side of the gel to the other.

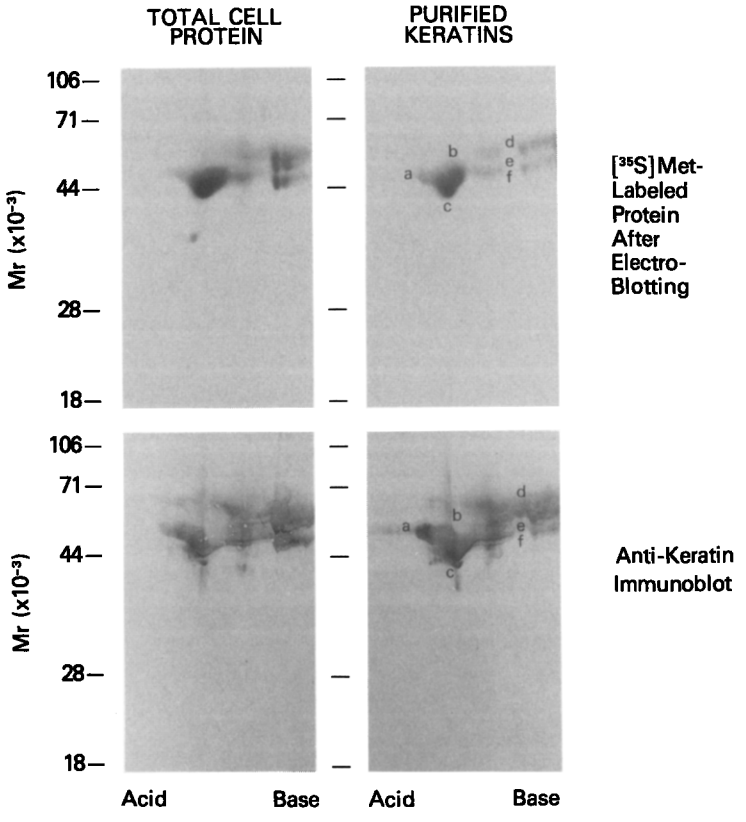

Fig. 3. Two-dimensional PAGE patterns of total cell proteins and purified cytokeratins of NHEK stained with anti-keratin antibodies. Two-dimensional gels of [^{35}S]methionine-labeled proteins were prepared as duplicates to those shown in Fig. 2. The proteins were electroblotted and the membrane was then stained with anti-keratin antibodies AE1 and AE3 as described under "Materials and Methods". The gels, containing the proteins that were not transferred, were fixed, stained, and prepared for fluorography. Exposure to the film was for 7 h. *Letters* identify proteins that have the same relative mobility in all of the figures. The "smiling-effect" seen in the second-dimension gels of the cytokeratin-enriched fraction probably was caused by a temperature variation from one side of the gel to the other.

p55^{RA} and p37^{RA} were seen in the total cellular protein but were absent from the cytokeratin-enriched fraction. Therefore, these proteins are probably not cytoskeletal proteins. The patterns of the proteins stained with Coomassie Brilliant Blue R-250 (data not shown) were comparable to the fluorograms of retinoylated proteins and of [^{35}S]methionine-labeled proteins exposed to the film for 2 h (Fig. 2). After a 12 h exposure to the film we found many [^{35}S]methionine-labeled proteins in the total cellular protein that were absent in the cytokeratin-enriched fraction (Fig 2). Additional evidence that many of the cytokeratins are retinoylated was the similarity of the immunoblots of proteins reacting with a mixture of type I and type II anti-keratin antibodies (Fig. 3) and the fluorograms of [^{3}H]RA-labeled proteins (Fig. 2, *bottom*).

We intentionally overloaded the two-dimensional gels shown in Figs. 1-3 to decrease the time to see the fluorograms of [^{3}H]RA-labeled proteins. Therefore, we did not obtain the high resolution separation of individual cytokeratins reported by others (19, 21, 23-27). Based on our patterns it seemed that phosphorylated and nonphosphorylated forms of type I keratins and type II keratins were retinoylated in NHEK. Additional studies should identify the retinoylated cytokeratins and determine whether there is a change in the pattern of retinoylation during the multi-step program of NHEK differentiation. Since changes in keratin expression accompanies squamous differentiation of NHEK, the differences in the retinoylation profiles shown in Fig. 1 suggest that the extent of retinoylation of some keratins are differentiation dependent.

In the human myeloid leukemia cell line HL60 about 90% of the RA covalently bound to protein is recovered as methyl retinoate after hydrolysis with 0.1 N KOH in CH_3OH for 2 h at 20°C (11). These results, as well as others, were consistent with retinoylation of HL60 proteins occurring with the formation of a thioester bond. In contrast, in NHEK only about 10% of the covalently bound RA was released after hydrolysis under the same conditions (data not shown) suggesting that in most of these retinoylated proteins RA probably is not bound by a thioester bond. This may not be surprising because the cytokeratins contain either no or few cysteine residues (28-30). The future objectives are to determine the nature of the covalent bond between RA and the cytokeratins and the identification of the cytokeratins that are retinoylated at different stages of differentiation. This information should contribute to a better understanding of the functional role(s) of retinoylation in epidermal cells.

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