Keratin Modifications in Epidermis, Papillomas, and Carcinomas during Two-Stage Carcinogenesis in the SENCAR Mouse

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ABSTRACT

To elucidate the role of keratin modification in tumor promotion, we investigated the keratin polypeptide patterns of mouse epidermis, papillomas, and carcinomas throughout an initiation-promotion experiment. The epidermal keratin modifications induced by repetitive 12-O-tetradecanoylphorbol-13-acetate treatments in both initiated and noninitiated mouse skin were essentially identical to those observed with a single 12-O-tetradecanoylphorbol-13-acetate application. These changes were even more pronounced in epidermal papillomas. In addition, the keratins of the papillomas displayed greater charge heterogeneity, particularly among the high-molecular-weight keratins (M, 60,000 to 62,000). As the experiment progressed, there appeared to be a selective loss of one group of high-molecular-weight keratins (M, 62,000) in some of the papillomas. Interestingly, the carcinomas that appeared at this time had significant reduction in both groups of high-molecular-weight keratins. In fact, the keratin profiles of carcinomas were very similar to the patterns observed in basal cells after a single 12-O-tetradecanoylphorbol-13-acetate treatment of adult epidermis. This may indicate that the program of keratin expression of a carcinoma becomes permanently fixed at a basal cell pattern. Changes in keratin patterns may serve as a biochemical marker of malignant progression in mouse epidermis.

INTRODUCTION

Mouse skin carcinogenesis has been divided into at least 2 stages: initiation, which is induced by a single application of a subthreshold dose of a carcinogen; and promotion, which is brought about by repetitive treatment with a noncarcinogenic tumor promoter. Treatment of mouse skin with these promoters, especially TPA, the most potent phorbol ester tumor promoter, causes inflammation, hyperplasia, and the induction of numerous other morphological and biochemical alterations (1, 11).

In the accompanying article (8), our laboratory has shown that TPA and several weakly promoting hyperplasogenic agents modify the epidermal keratins. These keratins, which are the major differentiation product of the epidermis, are thought to be involved in the regulation of cell shape and function as mechanical integrators of various cytoplasmic components (4–7, 12, 13). Some of the morphological and biochemical alterations attributed to TPA treatment may be due to the modification of the keratins, which may alter their normal cytoplasmic function. To gain understanding of the role of these keratin modifications in tumor promotion, we investigated the keratin polypeptide patterns of mouse epidermis, papillomas, and carcinomas during an initiation-promotion experiment by use of 2-dimensional gel electrophoresis. Because the keratin polypeptide patterns of papillomas, carcinomas, and uninvolved epidermis were found to be quite distinctive, changes in keratin patterns may serve as a marker of malignant transformation in mouse epidermis.

MATERIALS AND METHODS

Chemicals. TPA was obtained from Dr. P. Borchert (University of Minnesota, Minn.). DMBA was ordered from Sigma Chemical Company (St. Louis, Mo.). All reagents for SDS-polyacrylamide electrophoresis were received from Bio-Rad Laboratories (Richmond, Calif.). The isoelectric focusing ampholytes were obtained from LKB Instruments (Durham, N. C.). Nair was obtained from Carter Products (New York, N. Y.).

Treatment of Animals and Protein Extraction. Female SENCAR mice (7 to 8 weeks old) raised at Oak Ridge National Laboratory were shaved at least 2 days before treatment. There were 4 experimental groups: (a) a group of 100 animals which received multiple twice-weekly treatments of 1 μg TPA; (b) a group of 100 animals which were initiated with 10 nmol DMBA, followed 1 week later by repetitive 1-μg TPA treatments; (c) a group of 50 animals which received repetitive acetone treatments; (d) a group of 50 animals which were initiated with 10 nmol DMBA only. At certain intervals during the initiation-promotion experiment, 4 animals from each group were killed by cervical dislocation 48 hr after the last treatment. A chemical depilatory (Nair) was applied for 5 min to remove excess hair. The skins were removed, and the epidermis was scraped off with a razor blade. In some cases, the epidermis was separated by a trypsin flotation method (8), and basal cells were isolated from the epidermis as described by Green (6). If tumors were present, these were removed prior to scraping of the epidermis. Papillomas from 4 animals were pooled according to size: small, those measuring less than 0.3 cm; and large, those greater than 0.3 cm. Carcinomas were not pooled; instead, protein patterns from individual carcinomas were studied. Tissue samples from papillomas and carcinomas were fixed in 10% formalin for histological evaluation.

Epidermal proteins were isolated by a modification of the procedures used by Fuchs and Green (4, 6) and Dale and Ling (2). Prior to extraction, epidermal tumors were thoroughly chopped with scissors, and as much connective tissue was removed as possible from the tumors. The epidermis and tumors were placed into a solution of 4 M urea and 1 M phenylmethylsulfonyl fluoride and homogenized with a Polytron PT-10 homogenizer for 45 sec. The homogenate was centrifuged at 35,000 rpm for 30 min, and the pellets were resuspended in 2% SDS-10 mM DTT and then incubated at 37° for 1 hr. The 2% SDS-DTT extract was centrifuged at 35,000 rpm for 30 min. Because of the large amounts of proteins present in the tumors, we always used excess amounts of 4 M urea and SDS-DTT to ensure efficient extraction.
(usually 10 ml/tumor). The 4 M urea supernatant containing the more readily soluble proteins of the epidermis and the SDS-DTT supernatant containing the more insoluble proteins which were predominantly the keratins were then dialyzed against distilled water, lyophilized, and stored at −20°C until 2-dimensional electrophoresis was performed.

Two-dimensional Electrophoresis. The method of O’Farrell (9) was used. The isoelectric focusing dimension was run over a narrow acidic pH range (pH 4.0 to 6.0) in 5.5-mm glass electrophoresis tubes. This particular range was chosen because it would allow maximal separation of the individual keratins which have been shown to have isoelectric points within this pH range (12). The second dimension utilized 10% SDS-polyacrylamide slab gels. After electrophoresis, the proteins were fixed in 12% trichloroacetic acid and then stained in a solution of 0.1% Coomassie blue, 7% acetic acid, and 50% ethanol. Gels were destained in a solution of 7% acetic acid and 10% ethanol.

RESULTS

The more soluble proteins which were not cross-linked by disulfide bonds were removed with 4 M urea (5, 8). SDS-DTT was used to efficiently solubilize the epidermal keratins that were stabilized by disulfide bonds. Keratin changes that were observed in the SDS-DTT extracts could also be detected in the 4 M extracts which solubilized keratins not cross-linked by disulfide bonds. However, keratin modifications were best visualized using the SDS-DTT extracts because most of the nonkeratin proteins, not relevant to this investigation, were removed during the 4 M urea extraction. The majority of the SDS-DTT-extracted proteins resolved using our conditions of 2-dimensional gel electrophoresis have been shown to be the epidermal keratins based on their extractability, isoelectric point, molecular weight, ability to assemble into filaments in vitro, amino acid composition, peptide map, and immunological cross-reactivity (8). To aid comparisons, proteins were assigned numbers in the order of decreasing molecular weights. When proteins had similar molecular weights, letters were also assigned according to increasing acidic isoelectric points. A summary of numbers and molecular weights is shown in Table 1. No protein alterations were observed in mouse epidermis after multiple treatments with the solvent acetone. A representative 2-dimensional gel of the SDS-DTT-extracted proteins of acetone-treated epidermis is shown in Fig. 1A. The major proteins (Protein 1) were enhanced following TPA treatment of both noninitiated and initiated mouse epidermis (Fig. 1, B and C); however, unlike the papillomas (Fig. 2, A to C), the proteins in this region were not heterogeneous in charge. In other papil-

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lomas (Fig. 2 D to F), there appeared to be selective loss of the M, 62,000 protein component (1a and 1b). This was found not to be related to the size of the papilloma. However, as the experiment progressed, the loss of this group of keratins was observed more frequently than at earlier times. Although there were protein features which appeared to be distinctive for the papillomas, variations were noted among all the protein profiles of papillomas that were examined.

The protein profiles of the carcinomas which arose after 6 months of promotion were distinguishable from those of the surrounding epidermis and the papillomas. The protein feature which characterized the carcinomas was the significant reduction in both groups of high-molecular-weight keratins (M, 62,000 and 60,000) (Fig. 3, A to C). All the carcinomas contained Protein 4. As seen with the papillomas, Protein 4 was occasionally more predominant than Protein 3 (Fig. 3B).

To determine if the loss of the high-molecular-weight keratins upon malignancy was a feature limited to 2-stage carcinogenesis, we studied the keratin profile of a carcinoma induced by a complete carcinogenic dose of DMBA. The loss of the high-molecular-weight keratins was quite evident in the protein profile of the DMBA-induced carcinoma (Fig. 3D). Interestingly, Protein 4 was not very abundant; however, small amounts of the minor keratins were observed.

We also investigated whether the keratin pattern of purified basal cells was changed by treating adult skin with one application of TPA. We found that TPA induced a significant reduction in the high-molecular-weight keratins (M, 62,000 and 60,000) in adult epidermal basal cells (Fig. 3E). It is of interest to point out that the protein profiles of the basal cells isolated from TPA-treated skin were similar to those of the carcinomas, in that the high-molecular-weight keratins were substantially reduced. A more detailed study on the effects of various promoters and antipromoters on the protein profiles epidermal basal cells has been submitted elsewhere.

**DISCUSSION**

The keratin polypeptide patterns of mouse epidermis, papillomas, and carcinomas were found to be characterized by distinct keratin patterns. Although the keratins from papillomas and carcinomas were not characterized as extensively as the ones isolated from mouse epidermis (8), the fact that they had very similar solubility properties, molecular weights, and isoelectric points suggest that they are keratins.

The epidermal keratin modifications induced by repetitive TPA treatments in both initiated and noninitiated mouse skin were essentially identical to those observed with a single TPA application, which indicated that the epidermal response is not altered after prolonged treatment. In addition, these keratin modifications were found to be reversible when TPA treatment was discontinued.

The charge heterogeneity exhibited by the high-molecular-weight keratins was a consistent feature of papillomas. Since keratins have been shown to be phosphorylated (12, 13), it is possible that these multiple protein spots represent phosphorylated derivatives of the major keratins (Proteins 1, 2, and 2a) having different evidence obtained have elevated levels of protein kinases which are capable of phosphorylating the keratins. At early time points, the papillomas generally contained both groups of high-molecular-weight keratins (M, 60,000 and 62,000). A selective loss of the M, 62,000 keratin components was observed in some papillomas, becoming more frequent as the experiment progressed. We have previously demonstrated that TPA-treated adult epidermis and newborn epidermis contain both groups of high-molecular-weight keratins (8). The papillomas which also contain both groups of high-molecular-weight keratins resemble the newborn pattern except for the greater charge heterogeneity. Although no charge heterogeneity was observed, the keratin patterns of fetal epidermis at 17 days of gestation resembled that of some of the papillomas in which the M, 62,000 proteins were decreased or absent (data not shown). These changing keratin patterns may reflect different states of embryonic keratin gene expression that may occur during promotion. It is possible that the papillomas which have a selective loss of one group of keratins may represent a more advanced stage in the progression to malignancy. Preliminary data supporting this have been obtained in studies of both keratin patterns and GGT activity in individual papillomas. Recently, De Young et al. (3) demonstrated that malignant squamous carcinomas had elevated GGT activities, suggesting that GGT may serve as a marker of epidermal neoplasia. Klein-Szanto et al. (6) recently found that, in addition to carcinomas, a small percentage of papillomas have increased GGT activities. The majority of these GGT-positive papillomas were also found to lack the M, 62,000 keratins. Papillomas demonstrating elevated GGT activity as well as loss of certain keratins may be on the verge of malignancy.

The changes in the high-molecular-weight keratins were even more significant in the carcinomas, in which both the M, 62,000 and 60,000 components were substantially reduced. These protein alterations were shown not to be limited to carcinomas induced by 2-stage carcinogenesis; they were also observed in carcinomas induced by a complete carcinogenic dose of DMBA. Winter et al. (14) have also recently demonstrated that mouse skin malignant tumors specifically lack keratin components with molecular weights greater than 61,000.

The carcinoma keratin profiles were very similar to the patterns observed in basal cells following a single TPA treatment. This may indicate a basic defect in the program of differentiation in the carcinoma. By maintaining a basal cell pattern of gene expression, the cells of the carcinoma may be able to escape normal differentiation and growth controls.

Since the epidermis is composed of several distinct cell layers at different stages of differentiation in which protein changes in distinct epidermal populations might escape detection when extracts of the entire epidermis are studied, we thought that it was necessary to determine the effect of TPA on the basal cell protein pattern. The most striking feature in the protein pattern of the mouse basal cells after treating mouse skin with TPA was a decrease in the high-molecular-weight keratins which was also found in the keratin pattern obtained

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from carcinomas. By maintaining a basal cell pattern of gene expression, the cells of the carcinoma may be able to escape normal differentiation and growth controls. Several investigators have presented evidence which indicates that transformed epidermal cells do have basic defects in their program of differentiation (10, 15). Yuspa et al. (15) demonstrated that transformed epidermal cells continue to proliferate when exposed to a calcium environment which promotes terminal differentiation in normal cells. In addition, Rheinwald and Beckett (10) demonstrated that cell lines derived from human squamous cell carcinomas have a partial defect in terminal differentiation as measured by their survival and cornified envelope formation in semisolid media. However, it should be pointed out that Fuchs and Green (5) demonstrated that human basal cells synthesize mainly the small keratins and that during migration of the basal cells to the stratum corneum the synthesis of the large keratins was initiated.

In conclusion, changes in keratin patterns may serve as a biochemical marker for malignant transformation in mouse epidermis. Modification of the program of keratin expression in mouse epidermal basal cells may be one mechanism by which TPA promotes. TPA may induce changes that allows initiated basal cells to become fixed into a basal pattern of differentiation, resulting in a permanent growth advantage.

REFERENCES

2. Dale, B. A., and L
Fig. 1. Two-dimensional protein patterns of SDS-DTT extracts of mouse epidermis after 27 treatments with acetone (A), 27 treatments with 1 μg TPA (B), and DMBA initiation plus 27 treatments with 1 μg TPA (C). Protein patterns of animals which were removed for 12 days after 44 TPA treatments are also shown (D). The pH gradient of the isoelectric focusing dimension ranged between pH 4.0 and 6.0. Keratins migrated between pH 5.3 and 5.8. Molecular weights of the keratins ranged between 43,000 and 62,000. K, thousands.

Fig. 2. Two-dimensional protein patterns of the SDS-DTT-extracted proteins of papillomas arising during 2-stage mouse skin carcinogenesis. A, small papillomas obtained from initiated mice after 13 TPA treatments; B, small papillomas obtained after 16 TPA treatments; C, large papillomas obtained after 27 TPA treatments; D, small papillomas obtained after 27 TPA treatments; E, small papillomas obtained after 44 TPA treatments; F, large papillomas obtained after 83 TPA treatments. K, thousands.
Fig. 3. Comparison by 2-dimensional electrophoresis of the keratin patterns found in carcinomas during 2-stage carcinogenesis (initiation and promotion) with those found in a carcinoma induced by complete carcinogenesis (a carcinogenic dose of DMBA) and with those found in basal cells after TPA treatment. A, carcinoma obtained from an initiated mouse after 44 TPA treatments; B, carcinoma obtained after 57 TPA treatments; C, carcinoma obtained after 80 TPA treatments; D, carcinoma obtained after complete carcinogenesis with DMBA; E, keratin patterns obtained from basal cells isolated from adult mouse epidermis 48 hr after a single TPA treatment. K, thousands.
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