Transcriptional Control of High Molecular Weight Keratin Gene Expression in Multistage Mouse Skin Carcinogenesis

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ABSTRACT

Monospecific antikeratin antisera and specific complementary DNA probes were used to analyze expression of keratin genes in newborn mouse skin and skin papillomas and carcinomas by indirect immunofluorescence, immunoblotting, and in situ hybridization. Tumors were induced by initiation with 7,12-dimethylbenz(a)anthracene and promotion with 12-O-tetradecanoylphorbol-13-acetate. Type I epidermal keratin K14 protein (Mr, 55,000) is found in all living layers of the newborn skin but is most abundant in the lower strata. K1 (Mr, 67,000) and K10 (Mr, 59,000) proteins are predominantly suprabasal and K1 is processed in the stratum corneum. Transcripts for K14 were confined largely to the basal cell layer by in situ hybridization. Transcripts for K1 and K10 were highly expressed in suprabasal cells including the granular cell layer. In benign tumors, distribution of K14 protein is similar to that in newborn skin, while the abundance of K1 and K10 appears to be somewhat reduced although the tissue distribution remains suprabasal. Transcription of K14 is aberrant in benign tumors and transcripts persist throughout much of the suprabasal cell layers. Transcripts of K1 and K10 are normally distributed in papillomas but grain density is less intense than in newborn epidermis. Keratin expression in carcinomas is highly disturbed. K14 protein and transcripts are highly expressed in all strata in carcinomas while protein and transcripts for K1 and K10 are essentially absent. These results suggest that papilloma cells fail to respond to or generate account for the absence of Kl and K10 protein.

INTRODUCTION

For over 40 years, carcinogenesis research using mouse skin and other experimental models has revealed the multistage nature of cancer development. Early investigators were able to define a stage of tumor initiation, an irreversible change induced by the initial interactions of skin cells with carcinogens. The manifestation of the initiating event was recognized only as a result of subsequent exposures to tumor promoters, noncancerogenic agents which appear to induce reversible changes in the target tissue (1). In a number of target organs, the predominant tumor phenotype evolving from initiation-promotion protocols is benign (1). It now appears that subsequent genetic changes within the benign tumor cells are required to achieve the malignant phenotype (2-4). Classically, malignant conversion is scored by the appearance of a carcinoma, usually within the site of a preexisting benign lesion. Since the clinical appearance of carcinomas is likely to require many cell generations beyond the conversion event, analysis of the carcinoma will likely measure a number of genetic changes which may accumulate. The availability of a cellular marker which could recognize conversion at a preclinical stage would thus be helpful in understanding this aspect of carcinogenesis.

In the skin model, several markers have been identified which appear to distinguish carcinomas from their precursor lesion. For example, the activity of γ-glutamyl transpeptidase, an enzyme in hair follicles but not interfollicular epidermis (5, 6), is generally undetectable in benign tumors but histochemically detected in malignant tumors (7). Analysis of complementary DNA libraries from skin papillomas and carcinomas has revealed several sequences which are expressed specifically in the carcinoma (8, 9). Although the gene products for these sequences have not been definitively identified, they may represent useful markers pending complete identification of the protein. Another potential marker to distinguish benign from malignant cells is tranbin, a secreted protease which is also expressed in transformed cells in culture (10). However, tranbin is expressed in normal skin exposed to phorbol esters, a fact which would limit its usefulness as a marker in promoted animals.

Several laboratories have reported changes in keratin expression associated with malignant conversion in the mouse skin carcinogenesis model (11-13). Keratins are the major components of the cytoskeleton of all epithelial cells and the major differentiation product of skin. Keratins are encoded by a family of more than 20 genes which are differentially expressed in specific epithelia and in specific epithelial cell types within a given tissue. Keratin proteins can be subdivided into two major structural subgroups. Type I keratins have molecular weights of 40,000-60,000 with acidic PI values on isoelectric focusing gels. Type II keratins have molecular weights of 48,000-70,000 and migrate at a neutral or basic PI. In general, individual cells within a tissue express at least one type I and one type II keratin to form 10-nm cytoplasmic filaments. In epidermis, basal cells express primarily a Mr, 55,000 type I keratin (K14) and a Mr, 60,000 type II keratin (K5) as designated in the catalogue of Moll et al. (14). As cells enter their terminal differentiation program, the transcription of these keratins is diminished and a Mr, 67,000 type II keratin (K1) and a Mr, 59,000 type I keratin (K10) are expressed (15, 16). Expression of these latter keratins predominates in the suprabasal cell layers and these proteins are the major component of the stratum corneum, where post-translational processing also occurs (17). Analysis of mouse skin tumors by gel electrophoresis (11) has indicated that papillomas express, K5, K14, K1, and K10 and in addition contain keratins K6 and K16, which are expressed in skin and other tissues under conditions of hyperproliferation (14, 18).

Similar studies of mRNA (13) and protein (11, 12) have revealed a dramatic decrease in expression of K1 and K10 in malignant skin tumors. With regard to mechanism, data have been presented supporting a reduction in mRNA for K1 and K10 (13) or a reduction of K1 message translation (19) to account for the absence of K1 and K10 protein.

The cloning and sequencing of the keratin genes from mouse skin and human libraries have provided the essential tools to understand the regulation of these genes and to study changes in expression during carcinogenesis (see Ref. 20 for a review).
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Both structure and sequence data on the mouse epidermal keratins have been provided by this (21-24) and other (25) laboratories. Monospecific antibodies to unique peptides of the carboxy terminal portions of individual mouse keratin proteins have been generated. These antibodies have been useful probes to study the specific expression of individual keratin proteins within particular cell types (26, 27). Further gene sequence information has been useful to generate nucleic acid probes specific for the analysis of transcriptional activity by in situ hybridization (25, 28). In this report we use these tools to study the expression of keratins in both benign and malignant tumors from the epidermis to assess changes in their expression during tumor progression.

MATERIALS AND METHODS

Epidermal Tumors. Female SENCAR mice, approximately 7-8 weeks of age and in the resting phase of a hair cycle, were exposed to a single dose of 20 μg of 7,12-dimethylbenz(a)anthracene and promoted with 25 μg of 12-O-tetradecanoylphorbol-13-acetate. Promotion was continued for 10 weeks. The tumors examined in this study were excised at 30 weeks after initiation. At the time of sacrifice, portions of the tumor tissue were fixed in 10% buffered formalin, embedded in paraffin, and stained with hematoxylin and eosin. Other portions were rapidly frozen in Tissue-Tek OCT (Miles Laboratories) for frozen sections to evaluate by immunofluorescence and in situ hybridization (26, 28). Additional portions were quick-frozen in liquid nitrogen for protein extraction as previously described (29).

As a control, newborn mouse skin was similarly collected. Previous studies have shown that newborn skin and adult skin are similar with regard to keratin expression but the strata are more distinguishable in newborn epidermis, offering an advantage for morphological analysis.

Antibodies. Monospecific antikeratin antibodies were prepared as described (26, 27). Carboxy terminal peptides of K1, K10, and K14 were synthesized and injected into either rabbits or guinea pigs. Collected sera were assayed for antibody activity and the specificity of reactivity by Western blotting of epidermal extracts (26, 27). Antibodies were designated RK1, RK10, and RK14 for rabbit antibodies and GK1 and GK14 for guinea pig antibodies. The rabbit antibodies were purified by affinity chromatography using the synthetic peptides coupled to activated Sepharose (30). The availability of antibodies from two species provided the opportunity to study the coexpression of individual keratin peptides in single cells in the tissue. Single and double label immunofluorescence was performed as previously described (26, 31). For immunofluorescence studies the antisera were diluted as follows: GK1 (1/2000), GK14 (1/2000), RK1 (1/500), RK10 (1/500), and RK14 (1/500). The following dilutions were used for immunoblot analysis: RK1 (1/1000); RK10 (1/500); and RK1 (1/500).

In Situ Hybridization. 3'-Noncoding regions of complementary DNA clones for K1, K10, and K14 (23, 24) were subcloned into Gem 3 vectors as described by the manufacturer (Promega Biotech). Labeled riboprobes were generated by either SP6 or T7 RNA polymerase in the presence of 35S-labeled ribonucleotides. The specificity of the riboprobes was confirmed by performing Northern hybridization on nitrocellulose blots of epidermal RNA as described (29). In situ hybridization was performed on frozen tumor sections as described previously (28).

RESULTS

Analysis of Keratin Expression in Normal Mouse Epidermis. Frozen sections of newborn mouse epidermis were analyzed to establish the pattern of keratin expression in normal tissue. By immunofluorescence analysis (Fig. 1A) keratin 14 is strongly expressed in all basal cells and the proteins persist throughout the suprabasal cell layers but are absent in the stratum corneum. In addition, K14 is expressed in hair follicles. In contrast, K1 and K10 are primarily localized to suprabasal cells, and only a few V-shaped basal cells are noted to be positive for K1 and K10 (Fig. 1, B and C). Double label immunofluorescence with GK1 and RK10 reveals the consistent coexpression of K1 and K10 in a few basal and all suprabasal cells. Schweizer et al. (32) have previously detected basal cells expressing K10. Rare basal cells expressing only K1 have also been observed.3 Similar double label analysis confirmed that most K14 positive basal cells did not synthesize K1 or K10 but suprabasal cells were positive for K1, K10, and K14 (not shown). K10 is also seen in the stratum corneum while staining for K1 is markedly reduced or absent in the stratum corneum. Previous data suggest that the carboxy terminal portion of K1 is proteolytically released in the stratum corneum (26). Since these antibodies are directed against carboxy terminal sequences, the absence of staining likely reflects this posttranslational modification.

Because of the high stability of keratin proteins, dynamic aspects of expression can better be analyzed by evaluation of keratin gene transcripts by in situ hybridization (Fig. 1, D–F). K14 transcripts are strongly expressed in basal cells and in hair follicles, presumably in the outer root sheath, but diminish in the first suprabasal cell layer. Therefore, transcription of this gene is confined to the less differentiated cells although the proteins persist into the more differentiated cell layers. Transcriptional activity of K1 and K10 is strongest in the first suprabasal cell layer and persists throughout the upper strata, diminishing only in the uppermost granular cell layer. In agreement with the immunofluorescence studies, transcripts for K1 and K10 were also detected in the basal layer. However, the number of basal cells containing K1 and K10 transcripts (10–20%) is greater than the number containing K1 and K10 protein as detected with the antisera (5–10%). Therefore, after committing to terminal differentiation, at least some basal cells transcribe both K1 and K10 genes. However, an apparent lag may precede the appearance of K1 and K10 translation products. Hair follicles were negative for K1 and K10 transcripts.

Analysis of Keratin Expression in Epidermal Tumors by Immunoblotting. Cytoskeletal extracts were prepared from several papillomas and carcinomas, classified on the basis of gross morphological examination, and the integrity of the extracts was assessed by one-dimensional gel electrophoresis. Three papillomas and three carcinomas were selected for further analysis and their one-dimensional profiles are shown in Fig. 2. The most notable difference between the papilloma (Fig. 2, Lanes 1–3) and carcinoma (Fig. 2, Lanes 4–6) extracts is the absence of the M, 67,000 band in carcinomas. This band also appears diminished in extracts from papillomas P1 (Fig. 2, Lane 3) and P2 (Fig. 2, Lane 2). Other differences between the papilloma and carcinoma extracts are apparent in the M, 52,000–40,000 region of the gel; however, one-dimensional analysis does not allow accurate identification of these keratins with respect to the catalogue of Moll et al. (14). Since we have produced specific nucleic acid probes and antibodies to monitor the expression of keratin genes K1, K10, and K14, the following experiments will examine epidermal tumors for the expression of these genes at both the transcriptional and translational level. It should also be noted that numerous mouse skin tumors have been analyzed by the techniques described in the following experiments, and the data selected for presentation are representative of results accumulated over several years.

The cytoskeletal extracts shown in Fig. 2 were subjected to immunoblot analysis to detect keratins K14 (M, 55,000), K10 (M, 59,000), and K1 (M, 67,000) (Fig. 3). The position of the extracts shown in Fig. 2 are the mirror image of those shown

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3 D. R. Roop, unpublished observations.
Fig. 1. Analysis of keratin expression in newborn mouse skin by indirect immunofluorescence and in situ hybridization. Indirect immunofluorescence was performed with the following antisera: A, RK14; B and C, same section double stained with GK1 (B) and RK10 (C). Frozen sections of newborn mouse skin were hybridized with 32P-labeled RNA probes corresponding to K14 (D), K1 (E), and K10 (F). A-C, × 62.5; D, × 90; E, × 128; F, × 115.
in extracts from human epidermis. It is not known at the present time whether K10 and K11 are separate but closely related gene products or result from posttranslational modification. The concentration of K10 and K11 is approximately the same in the papilloma extracts shown in Fig. 3; however, extracts from newborn mouse epidermis contain predominantly K10 (26). As previously suggested from the one-dimensional analysis, the amount of K1 and K10 appears reduced in papilloma P1 relative to the concentration of K14, K1, and K10 in the other papilloma extracts.

Analysis of Keratin Expression in Epidermal Tumors by Double Label Immunofluorescence. In addition to immunoblot analysis, the tumors in Fig. 3 were examined by double label immunofluorescence with the K14, K10, and K1 antisera. The results obtained with three of these tumors are shown in Fig. 4. A–E represent results obtained with papilloma P3. The histological characteristics of this papilloma, as revealed by hematoxylin and eosin staining (Fig. 4A), are typical of those seen for benign tumors produced by initiation-promotion protocols. Double layer immunofluorescence staining patterns are shown in Fig. 4, B–E. B and C are the same section stained with GK14 (B) and RK1 (C), and D and E are the same section stained with GK14 (D) and RK10 (E). As observed for newborn skin, K14 staining is prominent in the basal cell layer and persists throughout most of the suprabasal layers of the highly stratified papilloma lesion. Unlike newborn epidermis, K1 and K10 staining of papillomas is confined to the suprabasal cell layers and not detected in basal cells. We have previously shown that proliferative cells persist in suprabasal regions of papillomas (37), and in many areas of the tumors the first and second cell layers are negative for K1 and K10. K1 staining is reduced in the most superficial layers, suggesting that this protein is processed as observed in the stratum corneum of newborn skin.

Results obtained with carcinoma C2 are shown in Fig. 4, K–O. The initial classification of this tumor as a carcinoma was confirmed by histological examination (Fig. 4K). L–O show double label immunofluorescence; L and M are the same section stained with GK14 (L) and RK1 (M), and N and O are the same section stained with GK14 (N) and RK10 (O). Virtually all of the cells of the tumor (excluding the stroma) are strongly positive for K14, whereas K1 and K10 staining is essentially negative. Therefore, these results are in agreement with the data obtained by immunoblot analysis of tumor C2. Cells staining positive for K1 and K10 have occasionally been observed in carcinomas (not shown). Interestingly, when positive cells are detected in carcinomas, K10 is detected more frequently than K1. The ability to perform double label immunofluorescence allowed us to confirm the epithelial nature of the tumor areas negative for K1 and K10 since these areas were positive for K14.

Results obtained for papilloma P1 are shown in Fig. 4, F–J. This tumor was initially classified as a papilloma on the basis of gross morphological examination; however, immunoblot analysis revealed that there were reduced levels of K1 and K10 in the cytoskeletal extract of P1. Interestingly, histological examination of this tumor revealed focal morphological variation (Fig. 4F). The periphery of the tumor contained well organized papilloma cells; however, the central area of the tumor was dysplastic. Double label immunofluorescence [G (K14) and H (RK1) are the same section and I (GK14) and J (RK10) are the same section] revealed that the periphery of the tumor stained positive for K1 and K10 but the central dysplastic area was negative. Both areas stained positive for K14. These results suggest that tumor P1 was examined at an early stage of conversion.

Analysis of Keratin Expression in Epidermal Tumors by in Situ Hybridization. In order to compare the distribution of transcripts for K1, K10, and K14 in papillomas with that observed for newborn skin (Fig. 1) and to determine whether the lack of expression of the differentiation-specific keratins in carcinomas was regulated at the transcriptional or translational level, in situ hybridization was performed with benign and malignant epidermal tumors (Fig. 5). A comparison of grain distribution for K14 (Fig. 5A), K1 (Fig. 5B), and K10 (Fig. 5C)
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Fig. 3. Immunoblot analysis of keratins from benign and malignant mouse skin tumors. Cytoskeletal extracts from the carcinomas (C1-C3) and papillomas (P1-P3) shown in Fig. 2 were subjected to electrophoresis, transferred to nitrocellulose paper, and reacted with antisera specific for K14 (M, 55,000), K10 (M, 59,000), and K1 (M, 67,000).

in papillomas with that observed in newborn skin reveals several differences. In particular, transcripts for K14 are much more abundant in papillomas and are not restricted to the basal layer, as in newborn skin, but extend well into the suprabasal layers. This is consistent with a diminished responsiveness of papilloma cells to differentiation signals whereby a keratin normally expressed predominantly in basal cells continues to be expressed in suprabasal layers. In general, the distribution of transcripts for K1 and K10 is similar in papillomas and newborn skin that grains are detected in the first and subsequent suprabasal cell layers. However, grain density for K1 and K10 transcripts in the first suprabasal layer seems to be somewhat less in papillomas than in newborn skin. This result is in agreement with the immunofluorescence data and suggests that the onset of maximal expression of the differentiation-specific keratin genes is delayed in the benign tumors. A very high level of K14 transcripts is observed throughout the epithelial areas of the carcinoma (Fig. 5D). Transcripts for K1 (Fig. 5E) and K10 (Fig. 5F) are usually not detectable in malignant cells, although occasionally positive cells have been observed. These data suggest that the absence of the differentiation-specific keratins in carcinomas is regulated at the transcriptional level.

DISCUSSION

The results reported here indicate that subtle changes in basal cell keratin gene expression occur in benign tumors while marked changes in suprabasal keratin gene expression are associated with malignant conversion during mouse skin carcinogenesis. In benign tumors, K14 transcripts are present to a greater extent in suprabasal cells relative to that seen in newborn skin. Increased expression of this keratin was previously noted in benign tumors analyzed by RNA blotting techniques (13), but those studies could not distinguish among enhanced transcriptional activity in all cells or selective expansion of a basal cell population expressing of K14 at normal levels. The present study suggests that enhanced expression which persists abnormally in suprabasal cells is the underlying mechanism that accounts for higher amounts of RNA for K14. The abundance of mRNA for K14 and its persistence could result from enhanced transcriptional activity and/or a change in message stability in the tumor cells. It is unlikely that the K14 probe is recognizing another closely related keratin mRNA since the probe is specific for a single RNA species on Northern blot analysis (not shown). However, the existence of another keratin gene, which is highly homologous to the K14 gene in both the coding and 3'-noncoding regions but contains different regulatory sequences which allow its expression in the suprabasal layers of benign and malignant tumors, cannot be excluded.

The persistence of expression of K14 in the upper regions of the benign tumor is compatible with benign tumor cells having a constitutive defect in their response to differentiation signals (37). Such signals would normally lead to a loss of K14 expression in the upper strata as seen in newborn skin. Since the factors which regulate keratin gene expression are unknown at this time, more mechanistic information cannot be gained by the experiments reported here. Recently it has been found that the pupoid fetus (pf/pf) mutant mouse has a phenotypic defect in epidermal differentiation which is lethal at birth in homozygotes (38). In these mice, K14 expression persists throughout the suprabasal layers (39), suggesting that such mice are genetically altered in their response to signals for epidermal differentiation or in the generation of these signals (40).

The availability of a marker which could detect malignant conversion in a benign tumor at an early stage would be valuable for assessing concomitant genetic alterations during conversion. The results obtained with papilloma P1 suggest that the loss of K1 and K10 occurs early in conversion; the loss can be detected by immunofluorescence with specific antibodies. This provides

C. Fisher and D. R. Roop, unpublished observations.

4 C. Fisher and D. R. Roop, unpublished observations.
Fig. 4. Analysis of keratin synthesis in benign and malignant mouse skin tumors by immunofluorescence microscopy. A-E, papilloma P3; F-J, papilloma P1; K-O, carcinoma C2. A, F, and K present the histology of the tumors as revealed by staining with hematoxylin and eosin. To simultaneously detect K14 and K1 or K14 and K10 in the same section, double label immunofluorescence was performed as follows: GK14 and RK1 were combined and used to stain the sections in B and C, G and H, and L and M; GK14 and RK10 were combined and used to stain the sections in D and E, I and J, and N and O. A, F, and K, × 36; B-E, G-J, and L-O, × 62.5.

A promising negative marker for studies with serial sections of tumors to analyze expression of other gene markers such as oncogenes or enzymatic changes during the conversion process reported in the accompanying paper (41). Our studies suggest that the loss of K1 and K10 in malignant cells is regulated at the level of transcription. The total absence of hybridizable mRNA detectable by in situ hybridization of histologically verified carcinoma foci is compatible with a transcriptional block in the expression of these genes in malignant skin tumors. In light of the previous suggestion that translatable message is present in extracts of certain transplantable squamous cell carcinomas (19), we cannot rule out that message stability is altered or transcripts are present below the level of the detection by in situ methods.

The absence of the differentiation-specific keratins in malignant mouse skin tumors resulting from initiation-promotion...
protocols has now been confirmed by three independent laboratories (11–13). Therefore, the loss of K1 and K10 protein and transcripts during malignant conversion seems a reproducible enough change to satisfy its use as a negative marker for malignancy in the mouse skin model. An exception has been noted but this was observed in tumors resulting from the transplantation of spontaneously transformed mouse keratinocyte cell lines (42). Even in cases where expression is detected,
there is a qualitative change in the tissue localization of the proteins (42).

The potential usefulness of these observations in the diagnosis of human cancers is less clear. Although human squamous cell carcinomas in general have reduced levels of the differentiation-specific keratins (14, 33, 34, 36, 43), a large percentage have minor, but significant, amounts of these proteins (34, 36). Therefore, to base the diagnosis of a human tumor as benign or malignant strictly on the presence or absence of the differentiation-specific keratins would be unwarranted. However, these specific reagents may be useful immunochemical tools for defining the state of differentiation of human squamous cell carcinomas. Thus at present, changes in suprabasal cell keratins may only be considered most reliable in the mouse model for carcinogenesis. In this model they may be particularly useful in the analysis of cellular and molecular aspects of malignant conversion.

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