Keratin protein identification in dimethylbenzanthracene-induced hamster cheek-pouch squamous cell carcinomas

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Individual keratin proteins were identified in 10 DMBA induced squamous cell carcinomas (SCC) of the hamster cheek pouch. SDS-polyacrylamide gel electrophoresis of water-insoluble cytoskeletal extracts from the tumor tissue demonstrated alterations in the protein distribution normal for the site. Immunoblot analysis with a broad spectrum polyclonal antikeratin antiserum identified the keratins in the preparations and confirmed changes in their distribution in the tumor preparations. The major keratin species for all the tumor tissues ranged in molecular weight from 45 to 57kd. The normal tissues had keratins with molecular weights from 45 to 73kd. The absence of high molecular weight keratins was a prominent feature in all the cancers. The histologic appearance of the tumors was varied but the distribution of the keratins was not correlated with the various histologies. The results demonstrate that changes in keratin gene expression occur in DMBA-induced cheek-pouch carcinomas but the precise alterations in the keratin proteins from those seen normally are not predictable.

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The production of SCCs in Syrian hamster cheek-pouch mucosa by topical applications of dimethylbenzanthracene (DMBA) is a well-studied oral carcinogenesis experimental system (1–3). The development of these tumors follows a predictable and reproducible course. This degree of control permits studies which would not be possible on human carcinomas. One characteristic which can be examined and compared is the molecular basis for the morphologic changes of the transformed epithelial cells.

The characteristic changes in the appearance of neoplastic cells reflect alterations in the cytoskeleton. Although several different elements contribute to the cytoskeleton; the group of epithelial-specific intermediate filaments known as keratins is of particular interest in epithelial neoplasms. Approximately 20 keratin genes exist; however, only a subset of these are expressed in any epithelial tissue, and the specific genes expressed are correlated with the pattern of differentiation of the epithelium (4–8). Studies of the keratin proteins present in human tumors have shown changes from the distribution usually observed in the normal tissue, but it is difficult to compare the keratin patterns since the natural history of the tumors is not known (4, 9–15). The hamster cheek-pouch carcinoma model permits changes in keratin gene expression to be assessed in tumors which result from a known etiologic agent and in a closely-controlled setting.

In the present study we sought to identify the individual keratins present in the tumor tissue and compare the distribution to normal cheek-pouch mucosa. The comparison was made to determine whether the induction of cancer was associated with a reproducible set of changes in keratin gene expression. Alternatively, the changes in keratin gene expression could be related to a secondary alteration occurring in neoplastic cells.

Material and methods

Tissue

Ten Syrian hamsters were painted 3 times per week with 0.5% DMBA in mineral oil for up to 11 weeks; and the animals maintained until a clincally observable tumor developed in the cheek pouch. Only one cheek pouch was painted with the DMBA-mineral oil solution; the opposite cheek pouch was treated with mineral oil only and served as an internal control. The animals were killed after development of the carcinoma and the tumor tissue was removed. The carcinomas were hemisected and one half was quick-frozen for eventual use in keratin extractions. The

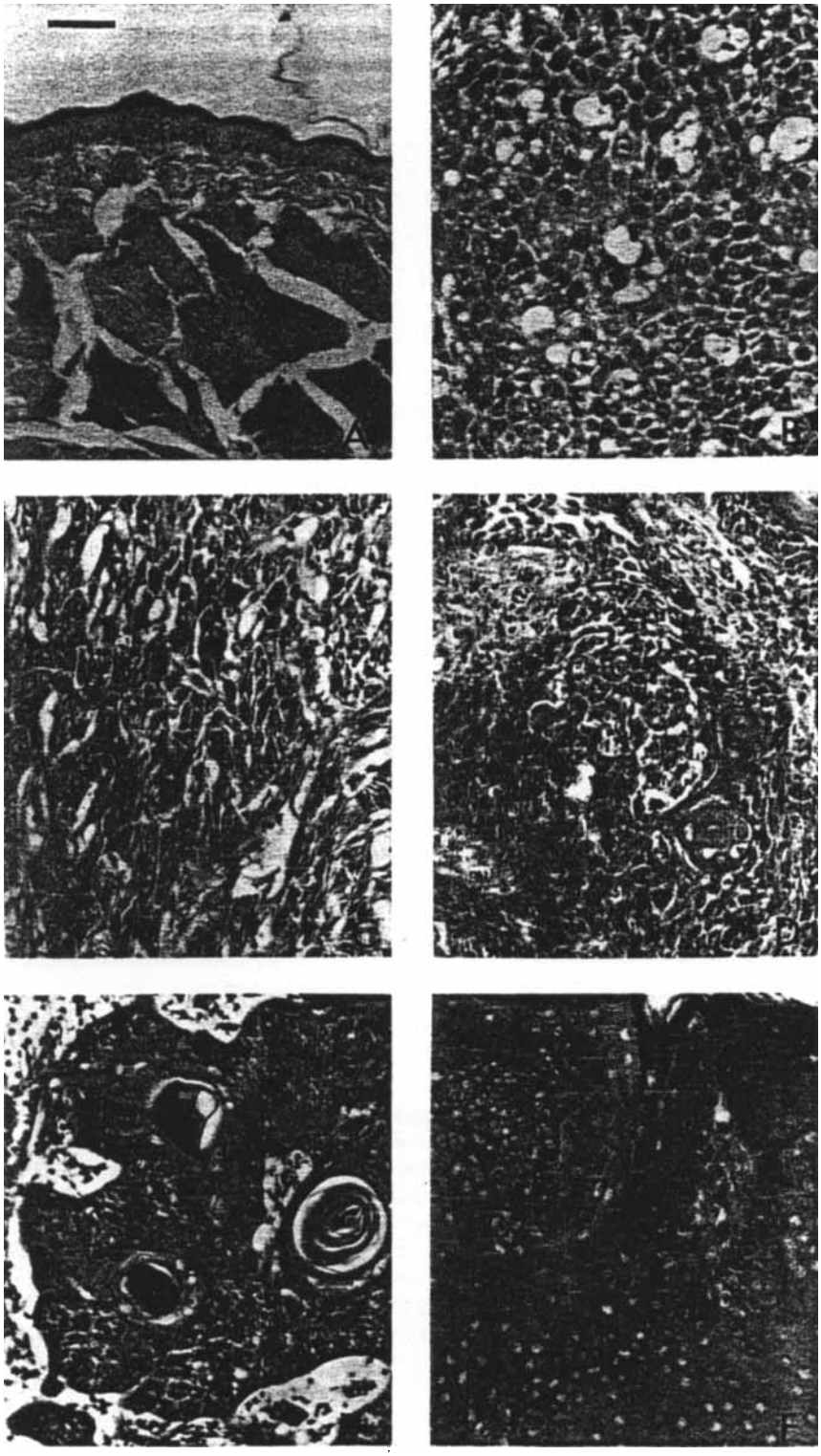

Fig. 1. Histology of normal cheek-pouch mucosa and DMBA induced cheek-pouch SCC. A. Normal cheek-pouch mucosa, B. Carcinoma with severe anaplasia, C. Anaplastic carcinoma with spindle-shaped cells, D. Carcinoma, moderate differentiation, E. Carcinoma, moderate-well differentiated with keratin pearl formation, F. Well-differentiated carcinoma with superficial invasion. All photomicrographs are at the same magnification (bar = 100 μ).

remaining half was fixed in neutral buffered formalin, processed, embedded in paraffin, sectioned at 5 μ and stained with hematoxylin and eosin. The control cheek-pouch mucosa, palatal mucosa and footpad epidermis were removed for use in histologic and keratin protein comparisons.

Cytoskeletal extraction

Water-insoluble cytoskeletal extracts were prepared from the cheek-pouch carcinomas, control cheek-pouch mucosa, palatal mucosa and footpad epidermis. The epithelium was separated from the underlying connective tissue mechanically following treatment of the tissues in 2M NaBr for 30 min at 37°C. The epithelial tissue was homogenized in 10mM Tris-HCl, 10mM EDTA pH 7.6. The homogenates were centrifuged and the pellets washed 3 times in the same buffer. The pellets were solubilized in 8M urea and 1% β-mercaptoethanol in 10mM Tris-HCl, 10mM EDTA pH 7.6 and sonicated 3 times for 15 s (16). The protein concentrations of the preparatations were determined (17).

Polyacrylamide gel electrophoresis

The protein extracts were electrophoretically resolved through 18 cm 10% polyacrylamide gels (30: 0.8 acrylamide: bisacrylamide) containing 0.1% SDS and prepared in 3mM Tris-HCl pH 8.8. Samples were electrophoresed for 1280 volt-h in 25mM Tris-HCl pH 8.8, 186 mM glycine and 0.1% SDS (18). Commercially purchased molecular weight standards (BRL) were co-electrophoresed on all gels. The gels were subsequently fixed in 10% methanol and 5% acetic aicd, and the proteins were visualized by silver staining. The molecular weights of the proteins were calculated by migration relative to the co-electrophoresed standards.

Immunoblot analysis

Immunoblot analysis of the electrophoretically separated proteins was performed (19). Identically-electrophoresed, unfixed gels were placed against a nitrocellulose membrane (S&S) and the proteins electrophoretically transferred in a Transphor Device (Hoefer) containing 25 mM Tris-HCl, ph 8.8, 192 mM glycine, and 20% methanol (v/v) for 160 volt-h. Following transfer the active sites on the membranes were blocked with 3% bovine serum albumin in 10 mM Tris-HCl, pH 7.6 and 0.9% NaCl for 2 h at 37°C. The filters were incubated overnight in a polyclonal rabbit antikeratin antiserum (a generous gift from Dr. Elaine Fuchs, The

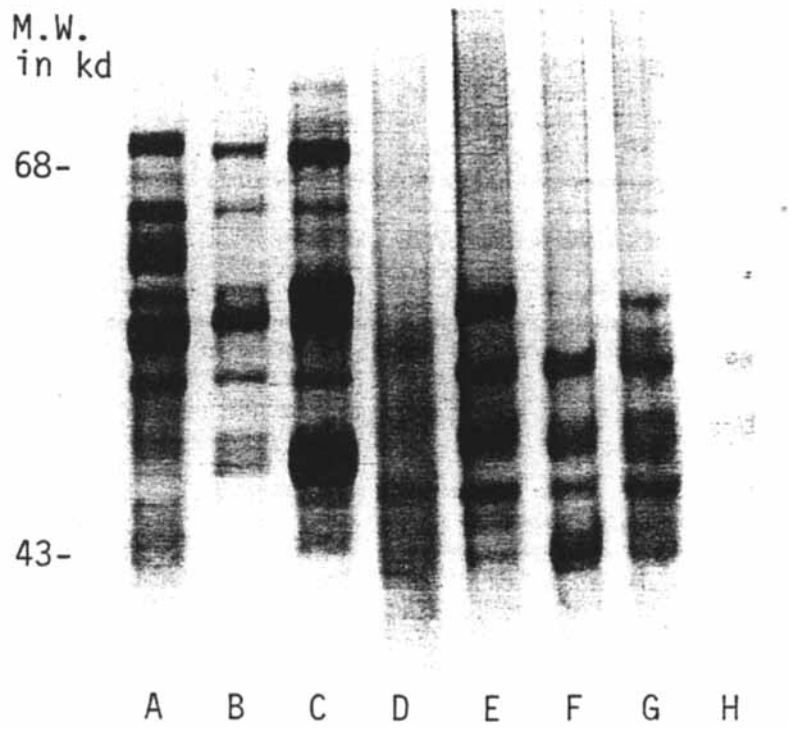

Fig. 2. SDS-Polyacrylamide gel electrophoresis of water-insoluble cytoskeletal extracts from normal and carcinoma tissues. A. Footpad epidermis, B. Palatal mucosa, C. Control cheek-pouch mucosa, D-H. Cheek-pouch carcinomas, protein extracts are from tumors shown in Fig. 1, B-F respectively.

University of Chicago) at room temperature. The filters were thoroughly washed and the antigen-antibody complexes visualized with a goat antirabbit IgG antiserum conjugated with horseradish peroxidase and 0.3 mg/ml diaminobenzidine and 0.003% H_2O_2.

superficial invasion. Histologic sections representative of these 5 histologic patterns are given in Fig. 1, B-F.

The water-insoluble cytoskeletal extracts were prepared from portions of all 10 tumors. When these protein preparations were electrophoretically resolved a limited number of different protein distributions were present. Fig. 2 includes examples of all the types of protein distribution found by SDS-PAGE and silver-stainng in the 10 tumor protein extracts. Fig. 2 also presents an example of the most common pattern of proteins resolved from each of the 5 histologic patterns identified in the 10 cheek-pouch SCCs shown in Fig. 1. The distribution of proteins was not specific for the histologic pattern of the tumor and, therefore, Fig. 2 does not present sets of proteins uniquely associated with a specific histologic appearance.

The major proteins present in all 10 tumor extracts were found in the molecular weight range between 45 and 57 kilodaltons (kd) (Fig. 2, D-H). The distribution of proteins present in the 3 normal tissues used for comparison was distinctly different than the distributions observed in tumor tissue protein extracts. In palate and cheek-pouch mucosa and footpad epidermis the molecular weight of the major proteins ranged from 45 to 73kd (Fig. 2, A, B, C). Therefore, one prominent change in the proteins isolated from tumor tissue when compared to normal epithelium was the absence of the higher molecular weight proteins. The molecular weight of the major proteins and the

Results

Tumors developed in the DMBA treated cheek pouches in all the hamsters from 9 to 11 weeks after initial application. The tumors were clinically present as exophytic masses either within or at the outer edge of the pouch and were easily observable. No tumors were present in any of the control cheek pouches either clinically or histologically. Histologic examination of the tumors revealed several different patterns of differentiation in the SCCs. Five different histologic patterns were identified in the 10 carcinomas. One tumor was identified as poorly differentiated with severe anaplasia, one as anaplastic poorly differentiated with spindle-shaped cells, one as moderately differentiated, 3 as moderate to well differentiated with keratin pearl formation and 4 as well differentiated with

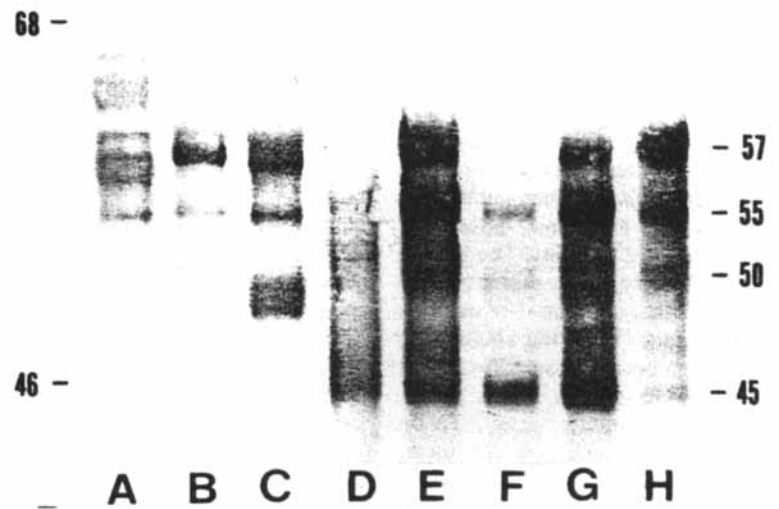

Fig. 3. Immunoblot analysis of cytoskeletal extracts using polyclonal antikeratin antiserum. A. Footpad epidermis, B. Palatal mucosa, C. Control cheek-pouch mucosa, D-H. Cheek-pouch carcinomas, protein extracts are from tumors shown in Fig. 1, B-F respectively.

method of extraction were consistent with that of keratins, however identification of the specific major proteins as keratins was required to permit characterization of the changes in these proteins in the tumor tissue.

The individual keratin proteins were identified by immunoblot analysis with a broad spectrum polyclonal antikeratin antiserum in protein extracts from all 10 tumors. The immunoblot analyses also identified a limited number of sets of keratin proteins in the tumor extracts and 4 different patterns were present. Fig. 2 has examples of the 4 patterns of keratins in the 10 tumor extracts and shows one example from each of the 5 histologic categories. The distribution of the keratins and the relative quantities were similar to the major proteins identified in the silver-stained polyacrylamide gels. The tumor tissue keratins were mainly between 45 and 57kd while the normal tissue keratins were in the 45 to 73kd molecular weight range. Both the number of keratins and the relative quantities varied between the 4 different keratin distributions. The specific distributions of the keratins in the tumor preparations were: 45, 46, 48, 52 and 54kd (Fig. 3D), 45, 46, 50, 54 and 57kd (Fig. 3E&H), 45, 50 and 54kd (Fig. 3F), and 45, 46, 50, 54, 57, 66, 70, 73kd (Fig. 3G). These could be compared with the keratin proteins present in normal footpad epidermis; 50, 54, 56, 57, 58, 62, 66kd (Fig. 3A), normal palate epithelium; 50, 54, 57, 58, 66, and 70kd (Fig. 3B) and normal cheek-pouch epithelium; 45, 48, 54, 57, 58, 64, 66, 70, and 73kd (Fig. 3C). All 10 tumor extracts had major keratins of 45 and 54kd (Fig. 3, D-H). Some of the tumor preparations also had similar quantitative levels of the 50 and 57kd keratins (Fig. 3, E, G, H). The remainder of the keratins present in the tumor preparations were present in much lower quantities. The only keratins present in the tumor tissue that were of a similar quantitative level as seen in the normal tissues were the 54 and 57kd proteins. The remaining keratins in the tumor extracts demonstrated either qualitative or quantitative differences from the proteins extracted from normal tissue. There were no keratins present in tumor extracts that were not present in at least one normal epithelium. There did not appear to be any correlation between tumor histology and keratin protein distribution and the 4 different distributions of keratins in the tumor tissue extracts were not unique for the specific histologic patterns of the carcinoma. Two tumors that were moderate to well differentiated with keratin pearl formation (Fig. 1E) had keratins as shown in Fig. 3G but the other tumor had a keratin pattern similar to Fig. 3E. Of the 4 tumors that were well differentiated with superficial invasion (Fig. 1F), 2 had the Fig. 3H keratin pattern while one had the Fig. 3G pattern and one the Fig. 3D pattern. Therefore, tumor histology could not be correlated with the keratins present in the tissue.

Discussion

The development of the transformed phenoytpe in a cell is dependent upon several changes in gene expression which endow the cell and its progeny with a different set of biologic potentials. The precise mechanism is unknown, however recently oncogenes have been identified whose expression is correlated with cellular transformation (20, 21). Although the precise changes in gene expression that result in the development of a neoplasm have not been determined the patterns of change in cell and tissue morphology can be used to diagnose a neoplasm and predict its clinical behavior. The characteristic changes in cellular architecture reflect both alterations in cytoskeletal gene expression and cytoskeletal assembly. Furthermore, the consistent patterns of histopathology suggest some relationship with the development of a neoplasm. Accurate comparisons of cytoskeletal elements are not possible in human tumors due to their unknown etiology and development. The DMBA-hamster cheek-pouch carcinoma system permits control during the induction of the neoplasm and allows changes in cytoskeletal gene expression to be analyzed and compared.

The epithelial-specific keratins represent one element of the cytoskeleton that presents unique opportunities for analysis in neoplastic epithelial tissues. Keratin gene expression has been shown to be closely correlated with the specific pattern of epithelial differentiation (4–8). Thus, different keratins appear to be associated with changes in epithelial cell shape. The changes in morphology in epithelial neoplasms might also be associated with differences in keratin gene expression. In this study we examined the keratins in DMBA-induced carcinomas in order to determine whether a reproducible pattern of changes in keratin gene expression was present.

The distribution of keratins isolated from the cheek-pouch carcinomas was distinctly different from that present in normal cheek-pouch mucosa. Most prominent was the absence of higher molecular weight keratins which have been associated with terminal differentiation of stratified squamous epithelium (4–7). A similar absence of these keratins has also been described in human tumors (4, 11). The presence of low molecular weight keratins has been demonstrated in cervical neoplasms and in cell lines derived from human tumors (13, 22). Interestingly, 2 of the cheek-pouch tumors which had keratin pearls also possessed higher molecular weight keratins as determined by immunoblot. While this finding indicated some relationship between the histology of the tumor and the present, consistent correlation between histology and keratins did not occur with all of the tumors. For example, identical distributions of keratins were present in a highly-differentiated carcinoma and in one with anaplastic spindle-shaped cells. This would seem to indicate that the keratins present were not correlated with the pattern of differentiation. Since the exact function of any keratin or set of keratins is not known, the patterns observed may still be related to a functional property of the transformed cells. The presence of the 45 and 54kd keratins in all of the tumor tissue preparations may be related to transformation, although the 54kd keratin was present in all the normal tissues and the 45kd keratin was present in normal cheek-pouch epithelium. Alternatively, they may represent the simplest set of keratins consistent with a functional intermediate filament in the cytoskeleton, and in the loss of gene control in the transformed cell these 2 keratins continue to be synthesized. The synthesis of keratins other than the 45 and 54kd may represent attempts by the transformed cells to complete a previously programmed pattern of terminal differentiation. This is supported by the presence of 2 additional keratins (50 and 57kd) common to 3 tumor preparations, which are identical with keratins present in normal tissues. Although hamster keratins are not as well-characterized as human, these 2 keratins may be analogous to the 50 and 58kd keratins which have been described as specific for stratified squamous patterns of differentiation in hu-

man epithelia (9). Despite the pronounced changes in keratins present, there were no keratins present in the tumor tissue which were not present in at least one of the 3 normal stratified squamous epithelia.

In this study we have demonstrated changes in the keratin proteins present in the hamster cheek-pouch carcinomas. Distinct differences in the distribution of keratins in the tumor tissue were present when compared to keratins extracted from normal mucosa. The differences in keratin distribution did not present a reproducible series of changes that were representative of a specific histology for the tumor tissue. Most prominent was the absence of higher molecular weight keratins in the tumor extracts. The absence of these keratins may be indicative of changes in the terminal differentiative pathway. The absence of the high molecular weight keratins may have prognostic value in future studies of the changes in keratin gene expression which occur during the early stages of development of hamster cheek-pouch carcinoma.

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