Cytokeratins in hamster cheek pouch epithelium during DMBA-induced carcinogenesis

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The pattern of keratin expression in hamster cheek pouch epithelium during 15-wk of DMBA-induced carcinogenesis was studied. The sequential changes in cytokeratins of premalignant and malignant tissues and comparative investigation of normal epithelial tissues were examined during a weekly sequential DMBA-induced chemical carcinogenesis. Keratin polypeptides of normal pouch epithelium appear in a molecular weight range of 43–67 kd and 5–6 proteins can be identified. The disappearance of high molecular weight keratin (61–67 kd) was observed from the 6-wk DMBA-treated premalignant group to the 15-wk DMBA-treated malignant group. An additional keratin polypeptide was noted initially on the 11th-wk-DMBA-treated group and remained to the 15th-wk-DMBA treated group.

Key words: carcinogenesis, oral; cytokeratin; DMBA; hamster; mouth, neoplasm.

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Keratins are the intermediate filament (IF) proteins of epithelia. Keratins consist of 20 distinct proteins between 40 and 70 kd and are classified into Type 1 (lower molecular weight with acid isoelectric point) and Type 2 (higher molecular weight with neutral to basic isoelectric point) (1). These IFs are markers of epithelial cellular origin and have been used in the pathological diagnosis of tumors (2–5).

In previous studies (6, 7), we found that the major keratin polypeptides in normal hamster pouch epithelium were affected in the pouches with squamous cell carcinoma. The cheek pouch squamous cell carcinoma lacked high MW keratin polypeptides from 61 kd to 67 kd. Osborn & Weber also found that the lower molecular weight keratins (43 to 58 kd) were present in all tumor tissue preparations (8). There have been different conclusions reported by prior workers on the disappearance or existence of low MW keratins in neoplastic tissues (9–11). However, most investigators agree with the absence of high molecular weight keratins in malignancy (12).

Winter *et al.* in studying the cytokeratin in the epidermis of rodents (13, 14) and in man (15), reported that the keratins of epithelial carcinomas exhibit a special pattern differing from that of their corresponding intact tissues as well as from benign tumors. They reported the lack of the large keratin polypeptides of type 2, with molecular weight higher than 61 kd. Viac *et al.* (16) and others studied different malignant human tumors and also demonstrated either an absence or a very low amount of the major protein band (MW 67 kd) present in normal human epidermis.

Löning *et al.* (11) studied keratin expression in human epidermis. They found that severe dysplasia and carcinoma in situ lesions were characterized by the high occurrence of premature single cell dyskeratosis associated with the presence of large keratins. However, Terry *et al.* (9) reported in the neoplastic vocal cord and in primary and secondary squamous cell carcinomas of the head and neck that the low molecular weight proteins were not readily detectable in normal stratified squamous epithelium but were present in the corresponding carcinomas.

The expression of keratins during experimentally induced carcinogenesis has not been studied (17). The specific keratin markers for neoplasms have been investigated in detail (6–8, 12, 17). The purpose of this study was to investigate the sequential changes in keratin expression during DMBA-induced hamster carcinogenesis. The keratin profiles in the course of fully malignant development were compared to normal cheek pouch, hard palate mucosal epithelium and foot-pad epithelia. This was done to define criteria that may predict the prognosis of the lesion

Material and methods

Induction of carcinoma

Sixty male adult (10–12 wk old) Syrian hamsters weighing 120–150 g were divided into 15 groups, each containing 4 animals. They were fed with a commercial diet and tap water. A 0.5% 9,10-dimethyl-1,2-benzanthracene (DMBA) in mineral oil was applied thrice weekly with a No. 4 camel's hair brush to the right cheek pouch of all animals. The untreated left pouch of all animals in each group served as a control. At the end of each week (3 days following the DMBA painting), one group of four animals was omitted from the painting and was killed by a lethal dose of diethyl ether. Pouches were inverted and the mucosa excised. Onehalf was frozen (−70°C) for SDS-PAGE electrophoeresis (18). The other half of each pouch was immersed in 10% buffered formalin, dehydrated in ascending alcohols, cleared in xylene and embedded in paraffin. Then sections were cut at 4 μ, stained with hematoxylin eosin and examined by light microscopy.

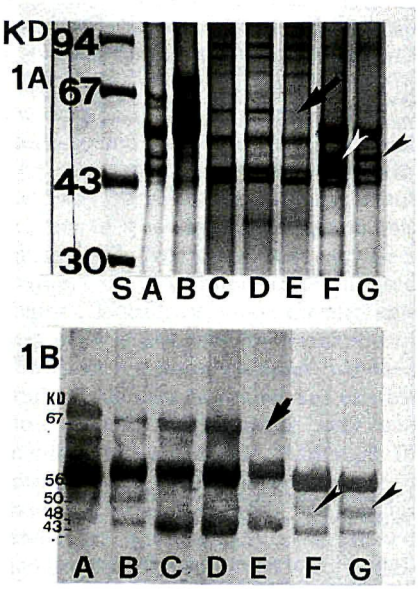

Fig. 1. A, SDS-PAGE keratin protein pattern of palate (A), foot pad (B), unpainted buccal pouch (C) and pouches painted for 5-wk (D), 6-wK (E), 11-wk (F), and 15-wk (G). Molecular weight standards (S). Note that keratin protein with molecular weight greater than 56 kd was lost started from 6-wk DMBA-painted group up to 15-wk (arrow). Additional band of keratin protein with molecular weight 50 kd was observed from 11-wk to 15-wk (arrow head). B, anti-keratin immunoblot of keratin proteins of foot pad (A), palate (B), unpainted pouch (C) and DMBA-painted pouches for 5-wk (D), 6-wk (E), 11-wk (F) and 15-wk (G).

Extraction of keratin

The method of extraction of keratin is adapted from AOYAGI *et al.* (19). The specimens for controls were taken from foot pad, buccal and palatal mucosa. The controls and the frozen half of the DMBA-painted pouches were each cut into small pieces (1 mm) and homogenized in a glass grinder with 10 mM EDTA solution. They were ground 90–120 times and centrifuged. After each were centrifuged three times at 8,000 g for 5 min, the pellets obtained were solubilized in the sample buffer containing 0.5 M Tris-HCl (pH 6.8), 2% sodium dodecyl sulfate, 8M urea, 1% 2-mercaptoethanol. Then, the samples were transferred into a 100°C water bath for 3 min, sonicated and centrifuged again, then frozen at −70°C until used for gel electrophoresis.

SDS-PAGE electrophoresis

Samples were analyzed by one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%) (SDS-PAGE) in a discountinuous buffer system according to LAEMMLI (18). The protein were stained with 0.25% Coomassie blue stain (R-250) for 20 min, and destained with acetic acid overnight and scanned with a densitometer ACD 2020 (Gelman Science Inc.). The molecular weight of the keratin proteins were calculated from a linear regression plot, being the best-fitting curve of the logarithm of the molecular weight, compared to the relative mobility of a commercially purchased standard proteins (Pharmacia: 94 kd phosphorylase b, 67 kd albumin, 43 kd ovalbumin, 30 kd carbonic anhydrae, 20.1 kd trypsin inhibitor) (20).

Immunoblot analysis

Immunoblot analysis was used to identify the keratin proteins separated by electrophoresis by the method described by TOWBIN *et al.* (21).

After electrophoresis, the unstained gel was immersed in the transfer buffer (20 mM Tris-HCl, 150 mM glycine, 20% methanol) for 30 min. The proteins were transferred to nitrocellulose membranes (Biotrace, pore size 0.45 um) in a Bio Trans Semidry electrophoretic transfer unit (Gelman Science Inc.) with a constant current of 0.8 mA/cm of gel for 2 h at room temperature. The nitrocellulose was first incubated in 1% Tween-20 in tris-buffered saline (TBS) for 1 h and washed twice with TBS containing 0.05% Tween-20.

Immediately following transfer, the nitrocellulose sheet was incubated in TBS for 1 h with 5% bovine serum albumin (BSA). Then the nitrocellulose was incubated in diluted 1:100 guinea pig anti-keratin (Sigma) for 2 h at room temperature, washed and then reacted with TBS diluted 1:500 goat anti-guinea pig IgG peroxidase conjugate (Sigma) for 2 h at room temperature. The nitrocellulose was washed five times with TBS containing 0.05% Tween-20, and then incubated with freshly prapared substrate solution containing 3,3-diaminobenzidine-HCl (0.05%) and 0.005% hydrogen peroxide in 50 mM TBS (pH 7.4) for 5–10 min.

Results

Gross examination of the DMBA-painted pouches revealed that the progress of the induced tumor began with erythema and erosion followed by repair. Later, there was thickened mucosa with a rough surface and whitish granular appearance (6–9 wk). Subsequently, various papillomatous growths developed. Squamous cell carcinomas

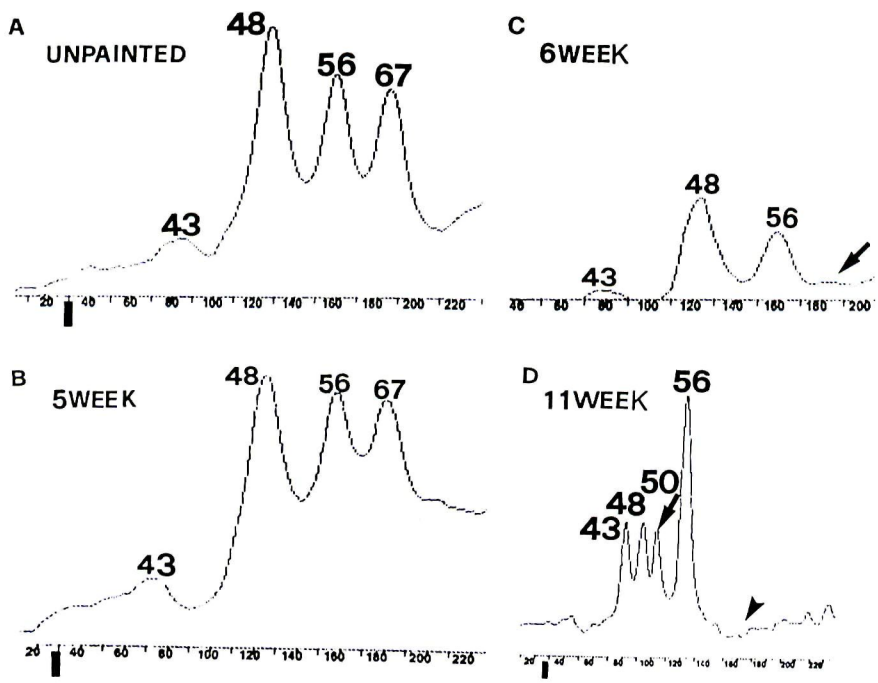

Fig. 2. Densitomer tracings of electrophoretic cytokeratin patterns in hamster cheek pouch epithelium. A, samples of unpainted cheek pouch with the main polypeptide bands of MW 43 kd, 48 kd, 56 kd & 67 kd. B, 5-wk DMBA-painted pouches showing similar bands to unpainted pouch. C, 6-wk DMBA-painted pouches showing the loss of 67 kd band (arrow). D, 11-wk DMBA-painted pouches showing the loss of MW 67 kd band (arrow head) and presence of an additional 50 kd band (arrow).

developed in the final stages (10–15 wk) of the lesion.

Microscopically, the change in the pouches began with an acute inflammatory reaction with engorged blood vessels and severe mucositis. Following the stage of repair, there was observed hyperkeatosis, acanthosis, mild dyskeratosis and lymphocytic infiltration. Following repair, deep invaginations of keratin or keratin plugs were observed to be prominent in the small papillomas. Then between 10–15 wk the pouch epithelial tumors increased in size, accompanied by areas of ulceration and tumor cell invasion into the underlying fibrous connective tissue and muscle tissue. These cells demonstrated hyperchromatism, pleomorphism, disorientation and increased mitotic index.

The SDS-PAGE electrophoresis revealed that individual composition of the major keratin polypeptides in the hamster food pad, palate and buccal pouch epithelium samples ranged from 43 kd to 70 kd (Fig. 1A). The immunoblot method with polyclonal antikeratin antiserum identified keratin polypeptides (Fig. 1B).

In the weekly sequentially DMBA-painted groups the cytokeratins showed various results as indicated in Figs. 1A, B. The keratin expression in the first week to the 5th wk DMBA painting groups was similar to that of the control group. The molecular weights of these keratins ranged from 43–67 kd, (Fig. 1A). However, from the 6th wk to the 15th wk DMBA painting, a lack of high molecular weight keratin was detected (Fig. 1A). A strong contrast in keratin expression occurred between the 5th week & 6th week (Figs. 1A, 2B, C). Keratin proteins above 56 kd were absent in premalignant and malignant epithelia. An additional low molecular keratin (50 kd) was expressed (Fig. 1A, 2D). This was observed from the 11th wk to 15th wk in the DMBA-treated pouches; the period when malignant transformation occurred.

Discussion

The keratin polypeptide pattern of unpainted hamster cheek pouch epithelium in our study had keratins expressed at 43 kd, 48 kd, 56 kd & 67 kd MW. This result was consistent with the keratins present in buccal mucosa reported by Clausen *et al.* (22, 23) and Mackenzie *et al.* (24).

The pattern of the high molecular weight cytokeratin expression appeared unchanged in the first 5 wk of the DMBA painting period. An absence of high molecular weight keratin in the DMBA-treated pouch epithelium occurred after the 6th wk of painting. An additional 50 kd keratin band was observed from 11-wk to 15-wk DMBA-painted group and was correlated with the histologic findings of epithelial proliferaton and malignant transformation. Neither the absence of 61–67 kd nor appearance of the 50 kd keratins expression was associated with the severity of the gross or microscopic features (or histologic differentiation) observed in the tumors.

The disappearance of 61–67 kd keratin polypeptides expression was not found to be associated with the severity of the gross or microscopic features observed in the tumor tissues. This finding is comparable to our previous observations that changes in keratin pattern in the tumor tissues could not be consistently correlated with the tumor histology (7). This may be due to the fact that the failure of the expression of high molecular weight keratin protein may possibly attribute to either the reduction of their correspondent mRNA or message translation (25, 26). Thus, the consequence of this molecular level defect in gene expression may not be reflected consistently with the state of differentiation observed at the miscoscopic level.

These changes support those reports of an altered expression of keratin polypeptides characterized by loss of high molecular weight keratin polypeptides, and the continued expression of keratins with low molecular weight during DMBA-induced carcinogenesis in hamster cheek-pouches (6, 7). In addition, the histopathologic finding of this stage of carcinogenesis was prominent as epithelial proliferation with mild to moderate dysplasia which was partially supported by McGrine (27) and Weiss's (28) work. In the context of cell differentiation, these findings suggest that there is an inherent disorder in keratin polypeptide synthesis consistent with incomplete maturation of squamous cells both in the hamster DMBA induced and human oral carcinoma.

Changes in the distribution of high and low molecular weight keratin proteins in the benign, premalignant and malignant lesions have been studied by many investigators (9–11, 13–16). The present work offers a description of the time-related changes in keratin proteins during chemical carcinogenesis. Further examinations with specific monoclonal antibodies could provide valuable information for the early detection of cancer in the hamster cheek pouches and possibly in human oral cancer.

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