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Expression of Epidermal Growth Factor Receptor, Polyamine Levels, Ornithine Decarboxylase Activity, Micronuclei, and Transglutaminase I in a 7,12-Dimethylbenz(*a*)anthracene-induced Hamster Buccal Pouch Carcinogenesis Model[1]

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ABSTRACT

The expression of epidermal growth factor receptor and transglutaminase type I, polyamine (putrescine, spermidine, and spermine) levels, ornithine decarboxylase activity, and micronuclei occurrence were assessed in the 7,12-dimethylbenz(*a*)anthracene (DMBA)-induced hamster buccal pouch model to elucidate the role and timing of changes in different growth and differentiation markers during carcinogenesis. DMBA (0.5%) in heavy mineral oil was applied to the right buccal pouch 3 times per wk for up to 16 wk; controls received heavy mineral oil alone. Hamsters were killed after 0, 4, 8, and 16 wk. Frozen tissue was chemically analyzed for polyamine levels and ornithine decarboxylase activity and was also used for immunohistochemical analysis of transglutaminase I. Paraffin-embedded sections were used for epidermal growth factor receptor immunohistochemical determinations and for micronucleated cell assays. Hyperplasia was detected by histological analysis at 4 wk, dysplasia with or without papillomatous changes at 8 wk, and squamous cell carcinoma at 16 wk. Epidermal growth factor receptor was not expressed in the normal buccal epithelial layer, at a moderate level in both the superficial keratin and basal cell layers in hyperplastic epithelium, and at very high levels in both dysplasia and squamous cell carcinoma. Transglutaminase I was expressed at a limited level in normal buccal mucosa, was expressed at a low level in the basal layer of hyperplastic lesions, was somewhat elevated in dysplasia, and was markedly enhanced in squamous cell carcinoma. Putrescine and spermidine levels and ornithine decarboxylase activity increased dramatically after 8 and 16 wk of DMBA. Micronucleated cells increased after 4 wk of DMBA treatment, that high level sustained during all stages of carcinogenesis. We suggest that these biological markers could be excellent intermediate end points in assessing the effects of various chemopreventive agents to be tested in the hamster buccal pouch model and in human clinical trials.

INTRODUCTION

Animal model systems have become increasingly important in the attempt to better understand the biology of carcinogenesis and improve the therapy of head and neck carcinomas. The Syrian golden hamster buccal pouch carcinogenesis model is probably the best known animal system that closely resembles events involved in the development of precancerous lesions and squamous cell carcinomas in human head and neck tissues. This model was first developed by Salley (1, 2) who experimentally produced a squamous cell carcinoma in the buccal pouch. Later, Morris (3) extended and standardized the procedure, enabling uniform reproduction of the lesions. In the last 25 yr, Shklar and others have extensively studied the model demonstrating the experimental efficacy of a variety of chemopreventive agents (4–13).

EGF-R[3] is a M_r 170,000 glycoprotein with an intrinsic tyrosine-specific protein kinase activity stimulated upon EGF binding (14). The sequence homology between the V-*erb*-B oncogene product and the cytoplasmic and membrane domains of the EGF-R has been reported previously (15). EGF itself has a potent mitogenic activity that stimulates proliferation of target cells in an autocrine fashion through its surface receptor (15). Gene amplification or overexpression of EGF-R has been observed in A431 human vulva squamous cell carcinoma cells, human glial tumors, human squamous cell carcinoma cell lines (16–20), and chemically induced malignant transformation carcinoma in the hamster cheek pouch model (21, 22).

In many experimental systems, one of the earliest indicators of genetic change is an increase in ODC activity and in levels of polyamines such as putrescine, spermidine, and spermine. For instance, ODC activity is rapidly induced by phorbol esters in mouse skin (23), rat tissues (24), and many cell types in culture (25). There are reports that phorbol esters and peptide growth factors induce ODC activity and cause alterations in polyamine levels in normal, preneoplastic, and neoplastic hamster fibroblasts and epidermal cells (26).

The development of malignancy often follows genomic damage that results in clonal expansion of cells with chromosomal abnormalities. Micronuclei are formed as a result of chromosomal nondysjunction after genotoxic damage (27, 28) and are a very sensitive index of genetic damage. Thus, we surmised that enumeration of micronuclei may provide a marker for the level of genetic damage induced by DMBA in hamster buccal mucosa.

Transglutaminases are a group of enzymes that covalently link peptide-bound glutamine to primary amines such as the ϵ-NH_2 of lysine (29). Epidermal transglutaminase, referred to as type I transglutaminase (30), appears to play an important role in the formation of cross-linked envelopes by keratinocytes, and it is likely that this enzyme can serve as a marker of squamous cell differentiation of epithelial tissues (30, 31).

In this study we analyze the expression of EGF-R, polyamine levels and ODC activity, micronuclei, and transglutaminase type I in the hamster buccal pouch model in order to determine their value as markers during carcinogenesis in this particular animal model.

MATERIALS AND METHODS

Animals. The Syrian golden hamster buccal pouch tumor model has been described previously (8). In brief, male Syrian golden hamsters

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[3] The abbreviations used are: EGF-R, epidermal growth factor receptor; EGF, epidermal growth factor; ODC, ornithine decarboxylase; DMBA, 7,12-dimethylbenz(*a*)anthracene; DAB, diaminobenzidine; DMSO, dimethyl sulfoxide; TBS, Tris-buffered saline [20 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl]; TDD, TBS supplemented with 0.3% DMSO and 4% dextran T-70.

were obtained from the Frederick Animal Research Facility, Frederick, MD, and at 4 to 6 wk of age were divided into two age-matched groups. The study group (application of DMBA in mineral oil) comprised 70 animals; the control group (application of mineral oil alone) comprised 30 animals. The animals were housed at room temperature with four pre plastic cage, each cage having wood chips for bedding. The light/dark cycle was 12/12 h. Water and laboratory chow (Rat Chow 5012; Ralston-Purina, St. Louis, MO) were given *ad libitum*.

DMBA (Sigma Chemical Co., St. Louis, MO) in a 0.5% solution in heavy mineral oil (U.S.P.) was painted on the right buccal pouch mucosa 3 times per wk in the study group. In the control group, heavy mineral oil alone was so applied. Animals were euthanized in a CO_2 chamber at 0, 4, 8, and 16 wk. Before an animal was killed, the right buccal pouch mucosa was grossly inspected to evaluate the premalignant lesion or tumor development and photographed. After euthanasia, the cheek pouch was excised, along with most of the underlying connective tissue and musculature. Tissues were subdivided and variously processed for distribution to each experiment.

Immunohistochemistry of EGF-R and Transglutaminase I. For EGF-R staining, buccal pouch mucosal tissues were fixed in 10% buffered formalin and embedded in paraffin upon excision. When the experiment was performed, tissue sections were deparaffinized in xylene and graded alcohol, followed by blocking of the intrinsic peroxidase activity with 3% H_2O_2 in methanol. Immunolocalization was performed with Vector's ABC kit (Burlingame, CA) per manufacturer's instructions. In brief, after an initial blockage with nonimmune horse serum, the sections were washed with phosphate-buffered NaCl solution, incubated with anti-EGF-R antibody, and then with biotinylated secondary antibody, followed by avidin-biotin-peroxidase conjugate, and finally incubated with the peroxidase substrate, DAB. Sections were counterstained with Mayer's hematoxylin and mounted in Eukit mounting medium. The anti-EGF-R monoclonal antibody used in this study was purchased from ICN Immunobiologicals, Lisle, IL, and is mouse IgG1 from C57BL/6 mice bearing the 29.1 hybridoma.

The immunohistochemical analysis of transglutaminase I was performed as described by Thacher (31). In brief, the tissue material was immersed in O.C.T. (Miles, Inc., Elkhart, IN) embedding medium and sectioned to 3 or 4 μm using a cryotome. The frozen sections were air dried for 2 h at room temperature and fixed for 3 min at −20°C in acetone. The fixed sections were washed twice for 5 min in TBS containing 0.02% NaN_3 (TBS/azide) and incubated for 30 min for blocking in TBS/azide supplemented with DMSO and dextran T-70 (TDD/azide) at 37°C. The sections were then incubated with a monoclonal anti-transglutaminase I antibody (IgG2a) from hybridoma B.C1 supernatant (30) with DMSO and dextran. Subsequently, secondary antibody (horse anti-mouse antibody) coupled to biotin (1:150 in TDD/azide) was incubated for 1 h at 37°C to detect anti-transglutaminase I. A 1:200 dilution of avidin and biotinylated peroxidase (Vector) in TDD without azide was used for 30 min at 37°C. The peroxidase was localized by incubating the sections for 10 min at room temperature in a solution consisting of 0.5 mg/ml of DAB, 1 mg/ml of nickel sulfate, and a 1:50,000 dilution of 30% H_2O_2. The slides were then washed in TBS, dried, and mounted for microscopic examinations.

Analysis of ODC Activity and Polyamine Levels. The tissue to be analyzed for ODC activity and putrescine and spermidine was frozen in liquid nitrogen upon excision and stored at −70°C. Extraction of the frozen tissue and analysis of ODC activity and polyamine levels were performed essentially as described by Harris *et al.* (32). Tissues from 3 animals of DMBA-treated and control groups were analyzed at each time point.

Micronucleated Cell Assay. The tissue preparation (paraffin embedded), staining techniques, and descriptive histology for the nuclear aberration assay have been previously described (33). Counting of micronuclei was performed by two investigators independently.

RESULTS

Tumor Induction Experiment. At the end of 4 wk of DMBA application, the mucosa of the right buccal pouch was rough, whitish, and thickened in 5 of 20 DMBA-treated animals. Microscopic examination showed hyperplasia with an increased keratin layer without mitotic activity in these five. The other 15 animals still had grossly normal mucosa. At the end of 8 wk, 7 of 20 animals showed papillomatous changes, with dysplasia (ranging from mild to severe in degree); the other 13 had hyperplastic changes. At the end of 16 wk of DMBA application, 15 of 17 animals had grossly evident squamous cell carcinoma, and the remaining 2 exhibited only dysplasia with papillomatous changes. The microscopic changes were illustrated in Fig. 1. The control animals did not show any histological changes during experiments.

EGF-R Expression. Immunohistochemical analysis of the expression of EGF-R was performed on samples from control animals and those treated with DMBA for 4, 8, and 16 wk. In control animals, the mucous membrane did not express EGF-R in the superficial keratin layer (Fig. 2*a*). The staining did not appear over time as mineral oil alone was applied. EGF-R expression, however, was more prominent in both the superficial keratin layer and basal cell layer in a focal manner in animals treated for 4 wk with DMBA (Fig. 2*b*). After 8 wk of DMBA, EGF-R expression was markedly elevated in dysplastic lesions (Fig. 2*c*), with focal staining again seen in both keratin and basal layers as after 4 wk. After 16 wk of DMBA, EGF-R was expressed at a very high level focally (Fig. 2*d*), with no expression in other areas of the same tumor section.

ODC Activity and Polyamine Levels. To evaluate the magnitude of proliferative activities during carcinogenesis in this animal model, we measured ODC activity and putrescine, spermidine, and spermine levels. The polyamine levels in animals treated with DMBA for 4 wk were not significantly different from those of control animals. However, after 8 wk of carcinogen treatment, putrescine and spermidine levels were, respectively, 3 times (663 ± 39 *versus* 178 ± 21 pmol/mg of protein) and 2 times (2701 ± 258 *versus* 1229 ± 92 pmol/mg of protein) higher than in controls. Interestingly, these increased levels correlated closely with the duration of DMBA exposure, not with histological changes, suggesting that polyamine levels are very sensitive indicators, their changes preceding histological changes.

After 16 wk of DMBA treatment, the putrescine level increased 20-fold (5410 ± 613 *versus* 245 ± 11 pmol/mg of protein) and the spermidine level remained elevated 2-fold (3840 ± 182 *versus* 1827 ± 115 pmol/mg of protein) over mean control values. There were no changes of spermine levels throughout the experiments (data not shown).

ODC activity was also consistently increased during carcinogenesis 3-fold (251 ± 6.1 *versus* 70.3 ± 3.5 pmol of CO_2/h/mg of protein) and 10-fold (915.3 ± 131.9 *versus* 94.7 ± 7.6 pmol of CO_2/h/mg of protein) after 8 and 16 wk, respectively, of DMBA. The summarized data are illustrated in Fig. 3. There were some insignificant differences in control values, presumably reflecting the aging process in this animal.

Micronuclei Incidence. After only 4 wk of DMBA treatment, the micronuclei count in the buccal mucosa was noticeably increased over controls, that frequency remaining roughly the same after 8 and 16 wk (Fig. 4). These results would seem to indicate consistent DNA damage by the carcinogen. Fig. 5 shows micronuclei near a nucleus in the buccal mucosa after 4 wk of DMBA, their number much elevated over the very low levels of micronuclei in control tissue at 4 wk (the count was generally less than 1 per 1000 cells in controls).

Immunohistochemical Staining of Transglutaminase I. Normal epithelium of the hamster buccal pouch was limitedly stained by anti-transglutaminase I antibody in the spinous layer beneath

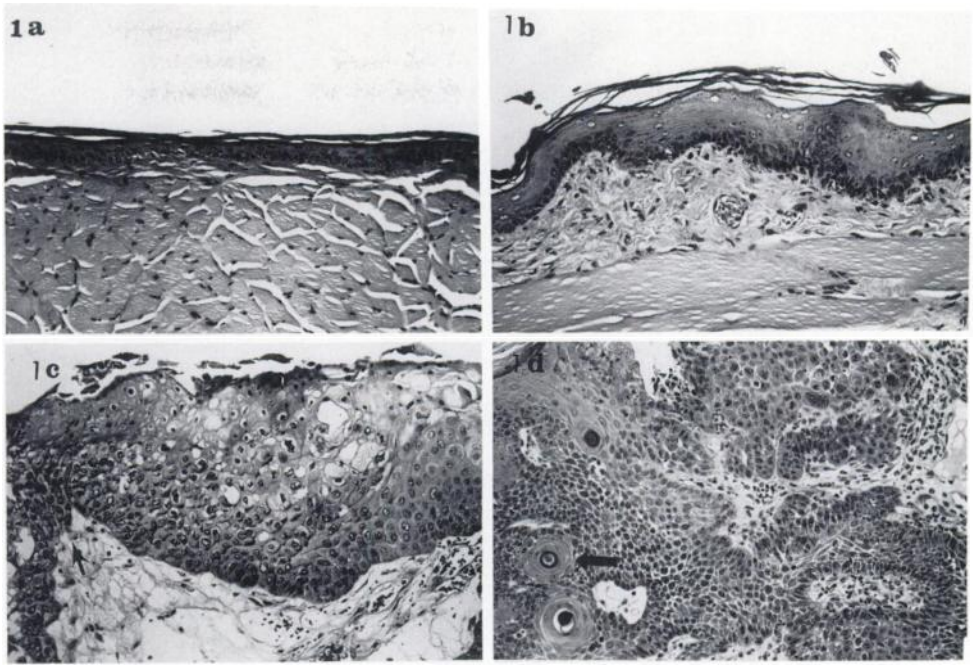

Fig. 1. Histological changes during DMBA-induced carcinogenesis. *a*, normal mucosal epithelium; *b*, hyperplastic changes of the keratin layer with proliferation of the basal cell layer after 4 wk of treatment; *c*, after 8 wk, proliferation of the keratin layer with prominent accumulation of basal cells, *arrow* indicating dysplastic changes; *d*, after 16 wk of treatment, an invading squamous cell carcinoma with prominent nuclei and scant cytoplasm, *arrow* indicating keratin pearl formation. H & E, × 100 (original magnification).

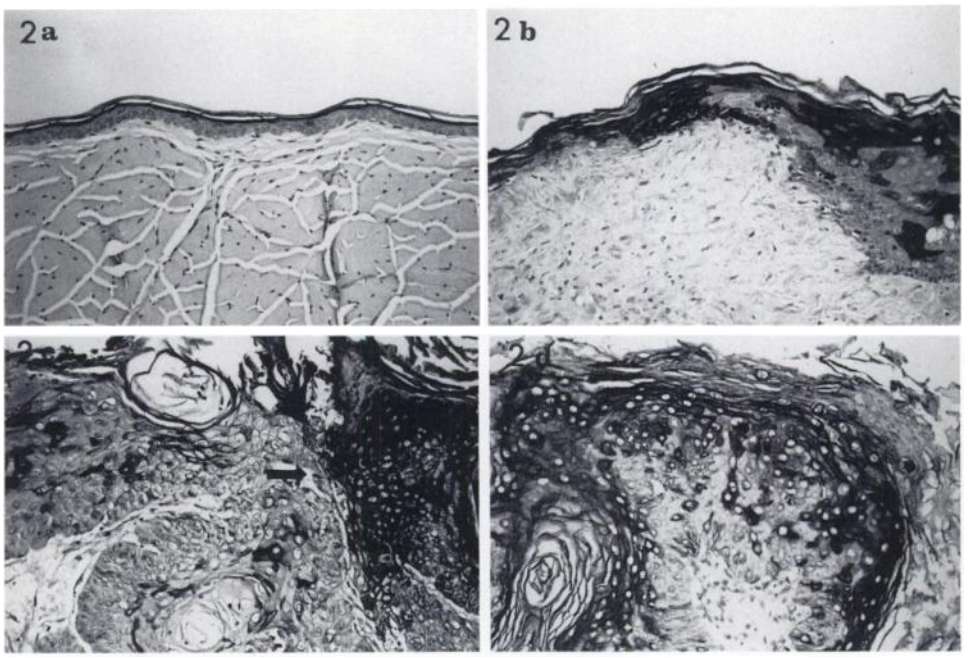

Fig. 2. EGF-R staining. In *a*, normal mucosa was not stained with EGF-R. In *b*, after 4 wk of DMBA treatment, expression of EGF-R increased in the superficial keratin layer and basal cell layer with a focal appearance. In *c*, after 8 wk of DMBA, there was intense expression of EGF-R in dysplastic areas (*arrow*). In *d*, after 16 wk of DMBA, squamous cell carcinoma shows high EGF-R expression, with focal increased staining. × 100 (original magnification).

the keratin layer (Fig. 6*a*). In contrast, after only 4 wk of treatment with DMBA, an increased staining at the hyperplastic basal cell layers was observed (Fig. 6*b*). Buccal mucosa after 8 wk of DMBA (Fig. 6*c*) demonstrated severe dysplasia, and staining with anti-transglutaminase I antibody was more intense and focal in nature. Further, staining was markedly enhanced in squamous cell carcinoma (Fig. 6*d*).

DISCUSSION

The hamster buccal pouch is an excellent model for the experimental induction of oral tumors by chemical carcinogens and is thus useful for testing chemopreventive and therapeutic agents (13, 34, 35). Little, however, is known about the biological and biochemical changes during carcinogenesis in this model. Wong and Biswas (21) and Wong (22) reported that mRNAs of c-*erb*-B1 and transforming growth factor α are overexpressed and that the genes are amplified in DMBA-induced squamous cell carcinomas and in a hamster cheek pouch carcinoma cell line (HCPC-1). Our results are in agreement with these results, as they indicate that squamous cell carcinomas induced by 16 wk of DMBA treatment express a higher level of EGF-R protein antigen than does normal mucosa. Furthermore, the squamous dysplasia that developed after 8 wk of DMBA treatment expressed EGF-R at a very high level. There are many reports on the overexpression, or amplification, of EGF-R in oral carcinomas and other tumor systems (20, 36–39). The more interesting finding in our study was that premalignant lesions, *i.e.*, squamous dysplasia (after 8 wk of DMBA), expressed EGF-R at levels as high as those found in squamous cell carcinoma. This finding suggests that altered expression of EGF-R may play an important role in transfor-

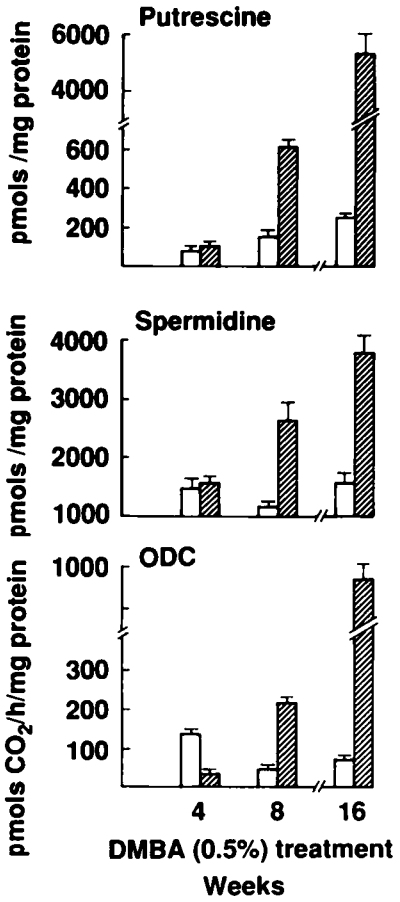

Fig. 3. Polyamine levels and ODC activity during carcinogenesis and in controls. The levels of putrescine and spermidine show no difference according to treatment with DMBA in mineral oil (▨) or mineral oil alone (□) after 4 wk. However, after 8 and 16 wk of DMBA putrescine and spermidine are markedly increased over controls. ODC activity has also increased steadily from 8 wk to 16 wk of DMBA. Frozen samples from 3 animals of the treated and control groups were analyzed at 4, 8, and 16 wk. *Columns*, mean; *bars*, SD.

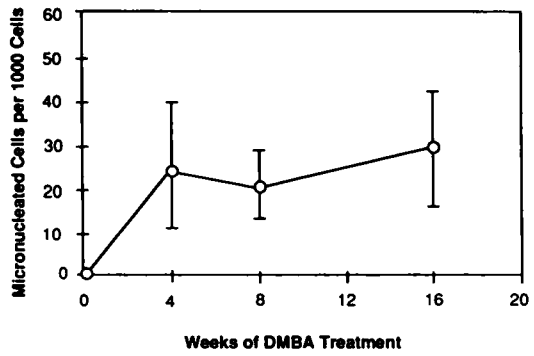

Fig. 4. Micronuclei count during carcinogenesis. The micronuclei count was highly elevated after only 4 wk of DMBA treatment. The 4-wk level defined a plateau that remained after 8 and 16 wk. *Points*, mean micronucleated cells/1000 cells; *bars*, SEM.

mation from normal through premalignant changes to squamous cell carcinoma.

Putrescine and spermidine levels and ODC activity were markedly increased over controls at 8 and 16 wk in DMBA-treated hamsters; notably, the polyamine increases correlated closely with DMBA exposure, anticipating histological changes. Polyamines are essential for the growth of cells, and rapidly proliferating cells have higher levels of polyamines than do slowly growing or quiescent cells (40). ODC is the initial and rate-limiting enzyme in the pathway of polyamine biosynthesis that catalyzes the conversion of ornithine to putrescine,

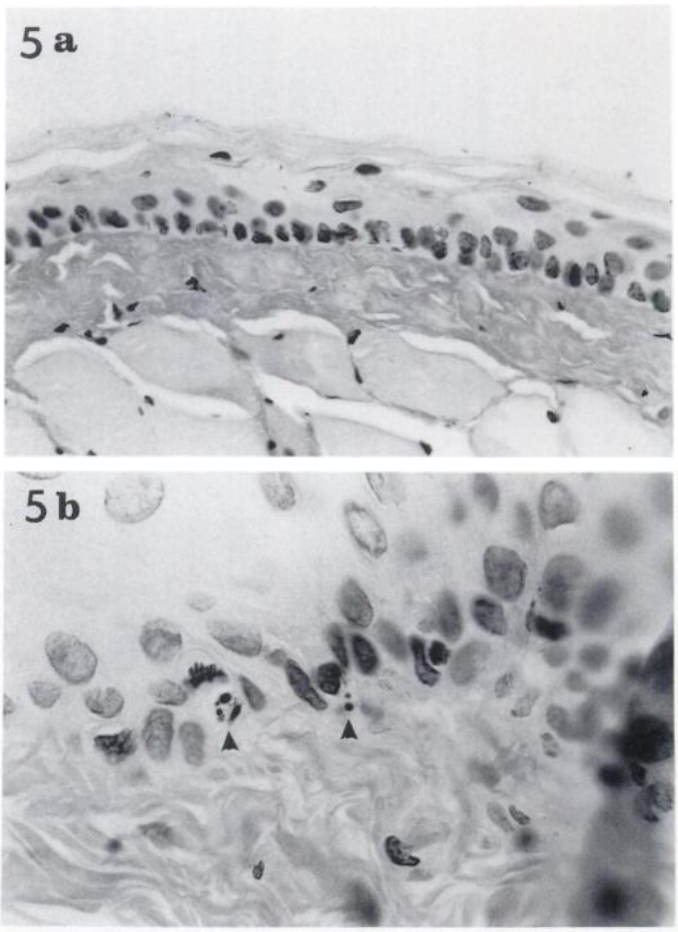

Fig. 5. A few micronuclei (*arrowhead* in *b*) are demonstrated after 4-wk DMBA treatment. Buccal mucosa of a control animal does not show any micronuclei (*a*). Feulgen and Fast green, × 1000 (original magnification).

and it is an index of degree of malignancy in certain tumors (see next paragraph) (41).

A variety of tumor promoters and tropic hormones also stimulate ODC and polyamines (42), and there is evidence that epidermal growth factor stimulates ODC activity and the rate of cell division (43). Our results are particularly interesting in that we found a correlation between EGF-R expression and polyamines/ODC activity for the samples treated with DMBA for 8 and 16 wk. ODC levels increase in proportion to the degree of malignancy in squamous cell carcinomas and brain tumors, particularly Grade IV astrocytoma and medulloblastoma (41). In that sense, our results indicate that the level of polyamines and ODC activity are well correlated with the degree of carcinogenesis from premalignant lesions to frank malignant squamous cell carcinomas in the hamster buccal pouch model as well. This phenomenon would be a very useful guide in chemoprevention studies in both animal models and clinical trials.

In this study, we assessed the specificity of induced nuclear aberration as a measure of the genotoxicity of DMBA to the buccal pouch of hamsters. Quantification of micronuclei has been used extensively in a mouse colon cancer model and used as a marker in some chemopreventive studies (44–46). The results of the present study suggest the findings pointing to the micronucleated cell assay as an excellent marker in evaluating the effects of chemopreventive agents in this animal model.

Transglutaminase I, an enzyme that catalyzes the cross-linking of protein precursors of the cross-linked envelope, nor-

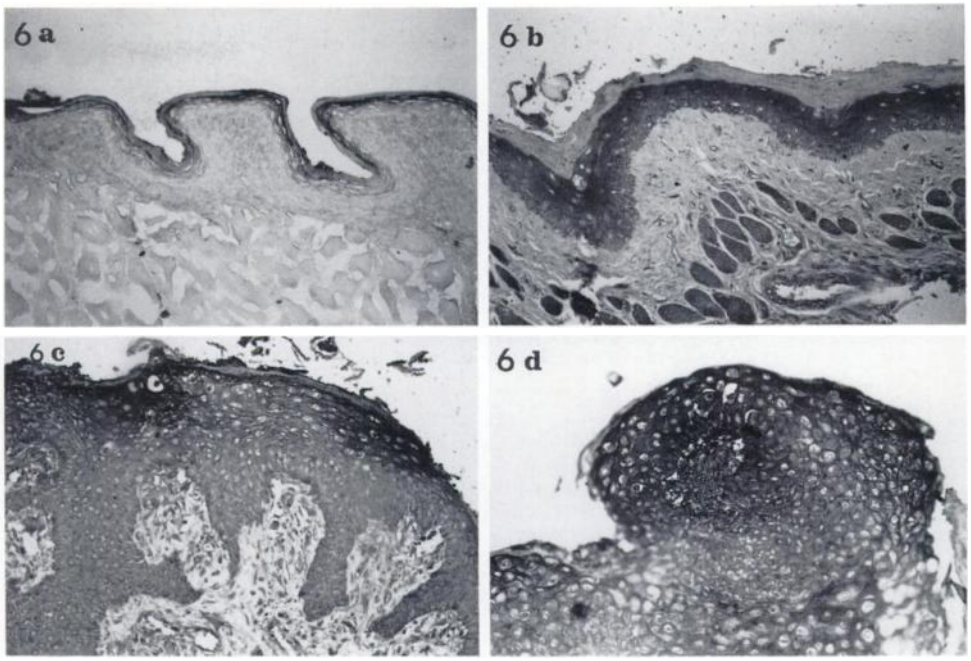

Fig. 6. Transglutaminase I staining. *a*, limited expression of transglutaminase I in normal (control) buccal mucosa; *b*, increased expression in hyperplastic cell layers after 4 wk of DMBA; *c*, moderately elevated staining after 8 wk of DMBA; *d*, markedly elevated expression after 16 wk of DMBA. × 100 (original magnification).

mally is expressed only during the latest steps of squamous differentiation such as occurs in epidermis (30, 31) or during tracheal squamous metaplasia (47, 48). A similar pattern of expression is observed in the untreated buccal pouch (Fig. 6*a*). After 4 weeks of DMBA treatment, however, there is greatly increased expression of transglutaminase I in intermediate and lower cell layers. The more extensive expression of transglutaminase I is similar to the precocious expression which occurs during epidermal hyperplasia, as a result of wound healing (49) or psoriasis (31). It would therefore be of interest to determine whether the agents having hyperproliferative effects alone would have the same effect. Our study shows the transglutaminase I is also strongly expressed during the later stages of DMBA treatment when cell morphology is markedly abnormal. Transglutaminase I expression may be a sensitive indicator for evaluation of chemopreventive agents in this model.

However, the pattern of expression of EGF-R and transglutaminase I is quite heterogeneous, probably because exposure to DMBA may select for a subpopulation of keratinocytes, either by enhancing or by altering the rate of proliferation (50), whereas there was a consistent association of highly inducible levels of ODC and polyamines during tumor promotion. Micronuclei increased dramatically in 4-wk samples treated with DMBA and were maintained at high levels during carcinogenesis. Of particular interest was the observation that all these biological markers were expressed at high levels either in hyperplastic or dysplastic stages during carcinogenesis in the hamster buccal pouch models.

We suggest that the biological markers presented here offer excellent parameters to follow during carcinogenesis in this animal model and could also be used as intermediate end points in assessing the effects of various chemopreventive agents in clinical trials.

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