An Animal Model for Oral Cancer

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ABSTRACT—Human head and neck squamous cell carcinogenesis (SCC) is a common malignancy that appears to be related to continuous exposure to putative carcinogens or promoters such as tobacco and alcohol. To understand the mechanisms of the development of head and neck cancer and to test the efficiency of new therapeutic approaches, the characterization of an animal model system is necessary. The cheek-pouch carcinogenesis model in Syrian golden hamsters is probably the best known animal system that is most closely comparable with the development of premalignant and malignant lesions in human oral cancer. Furthermore, it is one of the most well-characterized animal system models for SCC. Our first approach to understanding the cellular and molecular changes that occur in the hamster cheek-pouch carcinogenesis process was to compare this model to the mouse-skin system, in which a number of critical events have been well characterized. We examined the sequential expression of hyperplasia, micronucleated cells, ornithine decarboxylase activity, polyamine levels, transglutaminase I activity, epidermal growth factor receptor levels, expression of several keratins, γ-glutamyl transpeptidase, and nucleolar organizer regions. We suggest that these markers can be used to understand mechanisms of carcinogenesis and, in addition, can serve as alternative shorter end points in studies of chemoprevention. We also present preliminary molecular studies in the experimental oral model. We obtained a partial sequence of exon 2 of the Ha-ras gene and detected an $A^{182} \rightarrow T$ transversion in codon 61 in hamster cheek-pouch SCC induced by 7,12-dimethylbenz(a)anthracene. [J Natl Cancer Inst Monogr 13:55–60, 1992]

The cheek-pouch carcinogenesis of the Syrian golden hamster is probably the animal system process most comparable with the development of premalignancy and malignancy in human oral cancer. Furthermore, it is one of the most characterized models for squamous cell carcinomas (SCC). This was first developed by Salley (*1*), who produced an experimental oral SCC in the cheek pouch of the hamster. Later, Morris (*2*) standardized the procedures so that the experimental lesions could be uniformly reproduced. In the last 20 years, Shklar and coworkers have extensively studied the hamster cheek-pouch model and have performed a variety of chemoprevention experiments in this system.

The major advantages of the system as pointed out by Shklar et al (*3*) are the similarity between the hamster buccal pouch mucosa and the keratinizing human oral mucosa (in terms of histology, histochemistry, and ultrastructure), the absence of spontaneous carcinomas, the development of precancerous dysplastic lesions comparable to human oral leukoplakia, and the susceptibility of the tumor system to systemic influences, such as vitamins, hormones, and various drugs.

The carcinogenesis studies in the hamster cheek pouch are usually performed either by using multiple applications of 7,12-dimethylbenz(a)anthracene (DMBA) (usually 0.5% in mineral oil, three times weekly) or, occasionally, by using other carcinogens (*3,4*). The 0.5% dose of DMBA induced a hyperplastic response in the pouch epithelium after only a few applications, followed by the appearance of a variety of dysplastic lesions resembling human premalignant lesions after 6 to 8 weeks of treatment. Benign and malignant tumors (papillomas and SCC) started to develop after 10 weeks of treatment (*5*).

However, stages of carcinogenesis (initiation, promotion, and progression) have not been well defined in this system. To study the intimate cellular and molecular mechanisms involved in the genesis of oral tumors, a two-stage carcinogenesis animal model seems necessary in order to compare with the human oral cancer. The basic understanding of the pathogenesis of this tumor in the hamster cheek pouch is important for the development of new strategies of chemoprevention as well as for the study of chemopreventive agents and their possible mechanisms of action.

TWO-STAGE CARCINOGENESIS PROTOCOL

Tissue plasminogen activator (TPA) and benzoyl peroxide (BzPo) and other skin-tumor promoters produce well-defined, short-term effects on mouse skin, including sustained hyperplasia and proliferation. These short-term responses seem to be necessary events in the promotion process and are considered to be indicators of potential skin-tumor promotional activity of a compound (*6–9*). Although the phorbol ester is the most potent of the mouse-skin tumor promoters, it has been demonstrated that this compound has a very weak activity when applied in the hamster skin or cheek pouch (*10,11*). Furthermore, the lack of response of the cheek pouch to TPA has been confirmed in our laboratory (*12*).

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BzPo is a free radical-generating compound that has been demonstrated by Odukoya and Shklar to enhance the formation of tumors in the hamster cheek pouch when applied in combination with multiple treatment of DMBA (*13*). We analyzed a number of short-term markers of tumor promotion (keratins, hyperplasia, and nucleolar organizer regions [NORS]) in the hamster cheek pouch treated three times a week for 2 weeks with 40 mg of BzPo. Unlike in the mouse-skin model, BzPo was a more effective inducer of short-term markers of tumor promotion than was TPA (*12*).

CHEMOPREVENTION STUDIES

Chemoprevention has been studied in the hamster cheek pouch using a variety of natural and pharmacological agents. Many of these studies have focused on the use of vitamin A, vitamin E (alpha-tocopherol), and derivatives. Probably the most studied chemopreventive agent in the hamster cheek pouch has been vitamin E. Shklar showed that vitamin E significantly decreased tumor formation after 14 to 16 weeks of DMBA treatment; there were fewer tumors, and the tumors were smaller in size in the animals that received 10 mg of vitamin E twice weekly (*14–16*). Even more interesting were the experiments by Trickler and Shklar (*17*) using a lower dose of DMBA. Using 0.1% of DMBA instead of 0.5%, they found that tumor formation was completely inhibited by vitamin E.

The same group also studied the effect of vitamin E in established lesions of hamster cheek pouch (*18*). Hamsters that had been treated with DMBA for 13 weeks and developed SCC were then treated twice weekly for 4 weeks with 250 μg of vitamin E. In this study vitamin E was injected directly into the tumor-bearing pouch, and the treated hamsters were found to have a significant reduction in tumor mass. Microscopic examination showed that tumors were small and presented areas of degeneration with picnosis and inflammatory infiltration. Similar degenerative changes were also observed in leukoplakia.

The mechanism of the chemopreventive or the therapeutic action of vitamin E was not clearly established in these studies, but a study by Schwartz et al (*19*) showed that vitamin E prevented the depletion of Langerhans' cells caused by DMBA, suggesting an immunomodulating mechanism to explain, at least, the chemopreventive action of this compound.

Vitamin A and derivatives have also been extensively used in the hamster cheek-pouch model. A chemopreventive action of 13-*cis*-retinoic acid (10 mg given orally, twice weekly) was observed in animals treated with the standard DMBA protocol. Under this condition, the vitamin A derivative delayed tumorigenesis and reduced the size and number of SCC (*20*). Alteration in the distribution of Langerhans' cells in the epithelium and an alteration of the phytohemagglutinin blastogenesis was also observed, suggesting that the chemopreventive action of 13-*cis*-retinoic acid may also be mediated by the immune system (*21,22*).

One of the natural forms of vitamin A, beta-carotene, was also used in this system. In these experiments, in addition to the standard complete carcinogenesis protocol, a two-stage protocol using DMBA as the initiator and BzPo as the promoter was employed. Beta-carotene was shown to inhibit carcinogenesis in both protocols. In the two-stage protocol, beta-carotene appeared to act at both the initiation and promotion stages of carcinogenesis (*23*). Beta-carotene was also found to reduce the size and number of l-glutamyl transpeptidase (GGT)-positive foci in DMBA-treated hamsters (*24*). Later, Schwartz and Shklar (*25*) suggested that the effect of beta-carotene may not be mediated by the metabolic conversion of this product to retinoid, the biologically active form of vitamin A. In this study, they used canthaxanthin, which is a carotenoid that is not converted into vitamin A. In the hamster cheek pouch, canthaxanthin was a potent antitumor agent, although not as potent as beta-carotene.

Chemoprevention by natural extracts was also investigated in this system. The extract of Spirulina-Dunaliella algae was shown to be a potent tumor inhibitor when given either alone (*26*) or in combination with beta-carotene. These compounds were also effective in producing regression of established tumors in the hamster cheek pouch (*27*).

Onion extract, administered orally in the drinking water, was also an effective inhibitor of DMBA tumorigenesis. This extract reduced cell proliferation in a cell line derived from a DMBA-induced SCC (*28,29*).

Many other compounds have also been investigated as chemopreventive agents in the hamster cheek pouch. Inhibitors of prostaglandin synthesis such as aspirin, indomethacin, and ibuprofen (*30,31*) have been shown to exert a significant antitumorigenic effect when given daily by mouth concomitant with the cheek-pouch DMBA topical treatment. The effect of two protease inhibitors, Bowman-Birk inhibitor (BBI) and soybean trypsin inhibitor (SBTI), was also examined in this chemically induced oral carcinogenesis model (*32*). The soybean extract containing the protease inhibitor BBI suppressed DMBA-induced carcinogenesis, whereas SBTI did not. Immunoenhancing agents such as bacillus Calmette-Guérin (BCG) and levamisole were found to inhibit the development of hamster buccal pouch tumor (*33–35*). The delay in carcinogenesis was observed clinically and histologically. Phenanthrene (Phe) and, to a lesser degree, 1,4-dimethylnaphtalene (DMeN) were found to retard the development of carcinomas when applied topically in the DMBA-treated hamster cheek pouch (*36*).

GGT AND KERATIN STUDIES

Although the mechanism(s) by which chemical carcinogens induce cancer in this system are not clearly defined, there have been some biochemical and molecular studies described. Probably the best-studied event has been the induction of GGT, an enzyme that is not normally expressed in the hamster cheek pouch. Solt and Shklar (*37, 38*) showed that individual positive GGT cells or doublet

cells are detected histochemically as early as 3 days after the first DMBA treatment. After 3 weeks of treatment, they were able to detect GGT-positive intraepithelial cell foci (plaques) that appeared to be of clonal origin. GGT activity has also been demonstrated histochemically in areas of dysplasia, papillomas, and well-differentiated SCC. Odajima et al (*39*) have speculated from these results that probably the early GGT-stained cell populations are preneoplastic in nature.

The expression of different keratins has also been explored in this model. A profile of several keratins during experimentally induced carcinogenesis in hamster cheek-pouch mucosa was studied by immunohistochemical techniques (*40,41*). The antibodies used in these experiments were capable of identifying several groups of keratins, but they were unable to recognize individual members of this family of proteins.

We have explored the expression of keratins using immunostaining with monospecific antibodies and also by using a technique that allowed immunoblotting analysis of tissues embedded in paraffin (*42*). Monospecific antibodies against murine keratins were developed by Roop et al (*43,44*) using synthetic peptides corresponding to the DNA sequence of the 3′ nonconserved region of keratin cDNA. These antibodies recognize single members of the keratin family and can be used to investigate the presence or absence of specific keratins in histological sections. We have used antibodies against three keratins: K14, which is normally associated with proliferating cells (basal layer); K1, which is normally expressed in differentiated cells of the epidermis; and K13, which is expressed in differentiated cells of mucosa. The pattern of keratins for the hamster cheek pouch was consistent with that of the oral mucosa (*45*).

K14 was restricted to the basal layer, K13 to the suprabasal layer, and there was no demonstrable immunoreactivity with K1 by either immunohistochemistry or immunoblotting. However, K1 was expressed by the cheek-pouch epithelium in a time-dependent fashion in DMBA-treated hamsters. Concomitant with DMBA-induced hyperplasia, there were some topographical alterations in the distribution of K14. In this case, K14 was no longer restricted to the basal layer but was also expressed in differentiated cells. The same pattern was also observed in dysplastic lesions and in squamous cell carcinoma. Furthermore, expression of the K13 differentiation-associated keratin was preserved in this hyperplastic epithelium during all the stages of carcinogenesis, including either anaplastic or differentiated areas.

Alteration in the pattern of keratin expression appeared to be a common feature in the development of SCC in different systems (*44–50*). These alterations probably reflect abnormal differentiation patterns and are excellent tools with which to monitor the process of carcinogenesis.

ONCOGENES AND SUPPRESSOR GENES

The level of expression of several cellular proto-oncogenes was examined at different stages of DMBA-induced tumor development in this model (*51*). This study demonstrated overexpression of c-Ha-ras gene at a very early stage of tumor development. Conversely, expression of c-erbB was detected after 8 to 10 weeks of DMBA treatment and increased with the progression of the disease. Expression of c-myc and c-sis detected in control tissues remained unaffected, while c-fos gene activity could not be detected at any stage of tumor development. It has been suggested that the increased expression of the ras gene can be correlated with the initial transformation activity of the hamster cheek-pouch epithelial cells, whereas activation of the c-erbB gene can be correlated with the extensive proliferative and malignant phenotype of these cells in the intact animal.

Recent studies have indicated that the c-erbB proto-oncogene and the transforming growth factor (TGF-α) may be involved in the mechanism of chemical carcinogenesis in this system. The c-erbB gene that is the cellular gene for the epidermal growth factor (EGF) receptor has been found to be overexpressed in DMBA-treated pouch epithelium and in cheek-pouch tumors (*52,53*). Furthermore, this gene appears to be amplified in cell lines derived from SCC (*54*). TGF-α, but not EGF, was also expressed in the pouch tumors, suggesting a possible autocrine stimulation mechanism involving TGF-α and the EGF receptor.

The same authors have demonstrated that c-Ki-ras mRNA can be detected in DMBA-induced tumors, whereas no detectable c-Ki-ras mRNA can be found in the normal cheek pouch. The c-Ki-ras proto-oncogene has been found to be proliferation dependent, and Wong et al suggest that this proto-oncogene is quiescent in the normal cheek pouch, although its expression is associated with malignant transformation (*55*).

Previous experiments in other systems showed that DMBA-induced tumors presented a specific mutation in the codon 61 of the Ha-ras gene (*56,57*). Recently, our laboratory has investigated whether a similar mutation occurs in the hamster cheek-pouch SCC induced by DMBA complete carcinogenesis.

The normal sequence of a fragment of genomic DNA encompassing codon 61 of the Ha-ras gene was amplified by the polymerase chain reaction (PCR) using primers designed for a highly conserved region of the mouse Ha-ras-1 gene. The sequence of the amplified fragment was determined by direct sequencing technique and exhibited 83.3% and 87.5% homology with the corresponding human and mouse sequences, respectively. Homology at the amino acid level was identical for the three species. Paraffin sections of 11 squamous cell carcinomas of the cheek pouch were used to detect mutated Ha-ras alleles. DNA sequencing of the tumors showed that 10 of 11 tumors presented, and A→T transversion in the second position of codon 61 resulting in amino acid change from glycine to leucine (*58*).

Moroco et al (*59*) have postulated the existence of three suppressor functions in this model, which strongly suggests that inactivation of suppressor genes may be involved during the process of carcinogenesis. These results

raise the possibility of a cooperation between an activated ras gene and inactivation of a suppressor gene for the progression to malignancy, as was also recently proposed for the mouse-skin carcinogenesis model (*60,61*).

OTHER STUDIES

A number of biological markers was studied at different stages of tumor development in this model in order to define intermediate end points for assessing the effects of chemopreventive or therapeutic agents (*62*). EGF receptor had been shown to be expressed in the hamster normal cheek-pouch epithelium but was moderately present in the hyperplastic epithelium and strongly expressed in both dysplasia and SCC, whereas transglutaminase I, polyamine levels, ornithine decarboxylase activity, and micronucleated cells were increased during all the stages of carcinogenesis.

Changes of NORs in the hamster cheek-pouch chemical carcinogenesis had been demonstrated using a silver colloid technique (*63*). This technique provides information on the nucleolar activity of the cell (rDNA transcription) and has been considered as a potential marker of malignancy (*64–66*). The number and degree of activity of NORs were determined in DMBA-exposed epithelium and the resulting tumors. The percentage of all types of NORs presenting high-activity nucleoli increased during DMBA treatment, reaching the highest values in the SCC.

CONCLUSIONS

Induction of tumors in the hamster cheek pouch is a well-characterized model of chemical carcinogenesis that has been extensively used in studies of chemoprevention and chemointervention. In the last few years, several biological markers have been characterized in this model. These markers can be used to understand mechanisms of carcinogenesis and, in addition, can serve as alternative shorter endpoints in studies of chemoprevention. In the next few years, the hamster cheek-pouch carcinogenesis model is likely to provide new clues into the intimate mechanism of cancer development. Some molecular studies have already been performed in this system with promising results, and it is expected that in the near future, with the use of new technology, it will be possible to define precisely the molecular events related to the different stages of tumor development. This information will be instrumental in understanding human oral cancer and in developing strategies for cancer prevention and treatment.

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