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The Hamster Cheek Pouch Carcinogenesis Model

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Abstract The Syrian golden hamster cheek pouch carcinogenesis model is probably the best-known animal system that closely compares to events involved in the development of premalignant and malignant human oral cancers. Furthermore, it is one of the most well-characterized models for squamous cell carcinomas (SCCs). However, stages of carcinogenesis (initiation, promotion, and progression) have not been well-defined in this system. Basic understanding of the mechanism(s) of carcinogenesis in this organ is instrumental for the development of new strategies for chemoprevention and early chemointervention. To understand the important early events that occur in the hamster cheek pouch carcinogenesis model, we compared it to the mouse skin model, where a number of critical events have been well characterized. We determined that approximately 60% of the hamster cheek pouch SCCs have a mutation in codon 61 of the Ha-*ras* gene. We also established a two-stage carcinogenesis protocol in this model using a single dose of dimethylbenz(*a*)anthracene (DMBA) and multiple doses of benzoyl peroxide for 45 weeks. Twenty-five percent of tumors developed with this protocol had the same mutation in codon 61 of the Ha-*ras* gene, confirming that this mutation, as in the mouse skin model, is initiation-related. We examined the sequential expression of hyperplasia, micronucleated cells, ornithine decarboxylase (ODC) activity, polyamine levels, transglutaminase I activity, epidermal growth factor receptor (EGF-R) levels, keratins, γ-glutamyltranspeptidase (GGT), transforming growth factor-β_1 (TGF-β_1), leukoplakia, and carcinomas induced during carcinogenesis. A number of these important biological molecular and genetic markers could be excellent intermediate endpoints in assessing the effects of various chemopreventive agents to be tested in the hamster cheek pouch model and in human clinical trials.

Key words: intermediate biomarkers; chemoprevention; biological, molecular, and genetic changes; hamster; buccal pouch

Squamous cell carcinoma (SCC) is the leading form of malignancy in humans, affecting a wide variety of organs including skin, oral mucosa, pharynx, larynx, genital mucosa, *etc.* In the head and neck regions (oral cavity, nasopharynx and larynx), approximately 95% of tumors are SCC, accounting for 5% of all cancer in humans [1,2]. The etiology of this cancer has been linked to environmental factors, and epidemiological studies strongly suggest a role for chemical carcinogens [3,4]. Excessive use of tobacco and alcohol are also possible etiologic factors that may act synergistically to induce approximately 75% of SCC in the oral mucosa and larynx [4].

The nature of the chemical carcinogens involved in tobacco-related carcinogenesis has not been completely elucidated. A complex mixture of compounds, including not only genotoxic carcinogens, but also promoters, may play a role in these cancers [5,6].

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Since chemical agents appear to be the dominant etiologic factor in cancer of the oral mucosa and other areas of the head and neck, the use of carcinogen-induced animal models to study the mechanisms of carcinogenesis is warranted. The Syrian golden hamster cheek pouch carcinogenesis model is probably the best-known animal system that closely correlates events involved in the development of premalignant and malignant human oral cancers. Furthermore, it is one of the best-characterized models for SCC [7,8].

The major advantages of this model are similarity between hamster buccal pouch mucosa and keratinizing human oral mucosa, absence of spontaneous tumors, and susceptibility to systemic influences such as hormones, micronutrients (*e.g.*, retinoids, carotenoids, tocopherols, *etc.*), and others [9].

SCCs are induced in the buccal pouch using a complete carcinogenesis protocol that consists of multiple topical applications of a 0.5% solution of 7,12-dimethylbenz(*a*)anthracene (DMBA) in mineral oil by brush (Table I). Malignant tumors are preceded by a sequence of premalignant lesions similar to human leukoplakia and carcinoma *in situ*. Hyperkeratosis and chronic inflammation occur at 4–6 weeks, hyperkeratosis and dysplastic hyperplasia occur at 6–8 weeks, carcinoma *in situ* at 8–10 weeks, and invasive SCC at 10–16 weeks [10,11].

A two-stage carcinogenesis protocol was recently developed in our laboratory. The right pouch of 28 hamsters was initiated with a single dose of DMBA (0.5% in mineral oil). A second set of animals was initiated with vehicle only (mineral oil). Beginning 2 weeks after initiation, the hamsters were treated with either benzoyl peroxide (BzPo; 40 mg/200 μl acetone) or vehicle alone for 45 weeks (Table II). The presence of tumors or other lesions was investigated monthly by inverting the cheek pouch while the animals were sedated. As expected, there was a high incidence of tumors in the animals treated with DMBA and promoted with BzPo. No tumors were detected in animals given either chemical alone. Table III provides a schematic description of the different groups and results of this experiment. All tumors induced with this protocol were exophytic; histologically they presented verrucoid morphology. No flat dysplastic lesions were observed.

The hamster cheek pouch model has been used in numerous studies of chemoprevention. For example, the chemopreventive activities of vitamin A and some derivatives (13-*cis*-retinoic acid, β-carotene, canthaxanthin) were observed in animals treated with the complete (DMBA) carcinogenesis protocol. Also, vitamin E, either injected directly into the tumor-bearing pouch or topically applied, produced a reduction in tumor size as well as the number of tumors [12–14]. In Table IV, we show additional natural or pharmacological agents proven to have a chemopreventive effect in the hamster cheek pouch carcinogenesis model.

EARLY MARKERS OF CARCINOGENESIS

In the last few years, several biological and molecular markers have been characterized in experimental models for head and neck tumors. Using the complete carcinogenesis protocol, Solt *et al.* [27] demonstrated induction of γ-glutamyltranspeptidase (GGT), an enzyme not normally expressed in the hamster cheek pouch, as early as 3 days after the first DMBA treatment. GGT activity has also been demonstrated in areas of dysplasia, papillomas and SCC. Solt has speculated that the early GGT-stained cell populations are preneoplastic in nature.

The normal pattern of hamster cheek pouch keratins is consistent with that of the human oral mucosa. We investigated the immunohistochemical and immunoblotting pattern of two differentiation-associated keratins, K1 (67 kD) and K13 (47 kD) and a proliferation-associated keratin K14 (55 kD) with continuous DMBA treatment. We found that K1, which is not normally expressed in internal epithelium, was strongly expressed in the hyperplastic lesions. However, most dysplastic areas as well as carcinomas did not express this keratin except for small foci in well-differentiated SCC [28].

Normal expression of K13 was preserved during all stages of the DMBA complete carcinogenesis protocol [28], including anaplastic and differentiated areas. Expression of the proliferation-associated keratin (K14) was topographically altered during the process of carcinogenesis. After 2 weeks of DMBA treatment, K14 was no longer restricted to the basal layer, but was also expressed in differentiated cells. The same pattern was observed in dysplastic lesions and in

TABLE I. Complete Carcinogenesis Schematic

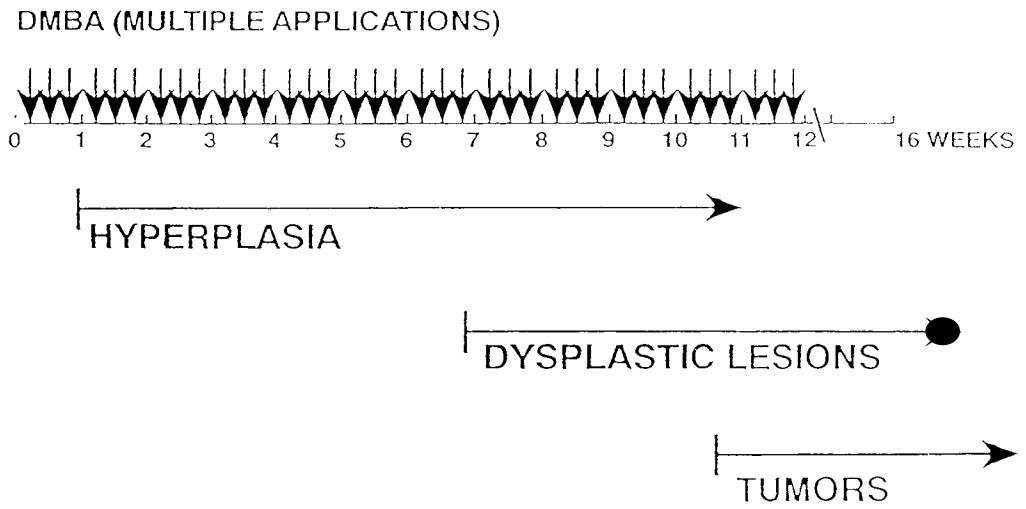

TABLE II. Two-Stage Carcinogenesis Schematic

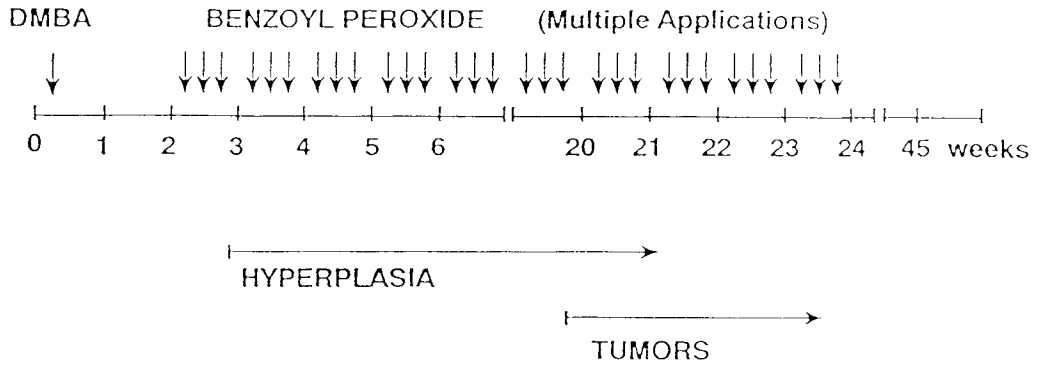

TABLE III. Two-Stage Carcinogenesis Protocol in the Hamster Cheek Pouch

	Initiation	Promotion	No. Hamsters	Incidence (%)	Average Tumors/Hamsters
Group I	DMBA 2 μmoles	BzPo 40 mg	28	60	0.9
Group II	DMBA 2 μmoles	Acetone	28	0	0
Group III	Mineral Oil	BzPo 40 mg	28	0	0
Group IV	Mineral Oil	Acetone	28	0	0

TABLE IV. Chemopreventive Agents in the Hamster Cheek Pouch Model

Agent	Author	Reference
13-*cis*-Retinoic acid	Shklar, G.	12,15
Vitamin E	Trickler, D.	13,14
β-Carotene	Suda, D.	16,17
Canthaxanthin	Schwartz, J.	18
Spirulina dunaliella	Schwartz, J.	19
Onion extract	Niukian, K.	20
Aspirin	Perkins, T.M.	21
Indomethacin	Perkins, T.M.	21
Ibuprofen	Cornwall, H.	22
Bowman-Birk inhibitor	Messadi, D.V.	23
Soybean trypsin inhibitor	Messadi, D.V.	23
Calmette-Guérin bacillus	Giunta, J.	24
Levamisole	Eisenberg, E.	25
Phenanthrene	Malament, D.S.	26
1,4-Dimethylnaphthalene	Malament, D.S.	26

SCC. Alterations in keratin expression appear to be a common feature during the development of SCC in different systems, and probably reflect abnormal differentiation patterns in the process of carcinogenesis.

The expression of transglutaminase type I, polyamine (putrescine, spermidine, and spermine) levels, ornithine decarboxylase (ODC) activity, and micronuclei incidence were also assessed in the hamster buccal pouch carcinogenesis model to elucidate the role and timing of changes in proliferation and differentiation markers during this process. Transglutaminase I was expressed at limited levels in normal buccal mucosa, low levels in the basal layer of hyperplastic lesions, somewhat elevated levels in dysplasia, and markedly enhanced levels in SCC. Putrescine and spermidine levels and ODC activity increased dramatically after 8 and 16 weeks of DMBA. Micronucleated cell incidence increased after 4 weeks of DMBA treatment; this plateau was maintained through the remaining stages of carcinogenesis [29].

Nuclear organizer regions (NORs) can be visualized in tissue sections by the silver colloid technique. This staining provides information on the nucleolar activity of the cell (rDNA transcription) and has been considered a potential indicator of the degree of malignancy. We analyzed NORs in DMBA-induced benign, premalignant, and malignant lesions of the hamster cheek pouch. The number and degree of activity of NORs were determined in a minimum of 100 cells/section. The percentage of gathered-type NORs with high-activity nucleoli increased in the pouch epithelium during DMBA treatment and reached the highest values in the malignant tumors. The percentage of dispersed-type NORs also increased in the malignant lesions. However, the absolute number of NORs was not affected by DMBA treatment. These results suggest that DMBA-induced modification of NOR activity occurred during the early stages of carcinogenesis, showing the potential of this model for studying NOR alterations in neoplasia [30].

GROWTH FACTORS AND GROWTH FACTOR RECEPTORS

Transforming growth factor-α (TGF-α) and epidermal growth factor receptor (EGF-R) have

been demonstrated in most SCCs, and the expression of TGF-α has been associated with malignant transformation. Wong *et al.* [31] showed the presence of TGF-α and EGF-R mRNAs in chemically transformed hamster oral keratinocytes, but TGF-α mRNA could not be detected in normal hamster cheek pouch epithelium, suggesting that an autocrine stimulation mechanism might be involved in tumor cells. Using a monoclonal anti-EGF-R antibody, we also showed an increase in the expression of this antigen during hamster cheek pouch carcinogenesis [29].

There is no information about a possible role of TGF-β_1 in hamster cheek pouch carcinogenesis, in spite of the fact that alterations in the expression of this cytokine have been found in several human and experimental tumors [32, 33]. TGF-β_1 is a regulator of cell growth and differentiation in squamous epithelia and other tissues. While inhibiting the proliferation of a number of epithelial cell types, TGF-β_1 usually stimulates the proliferation of mesenchymal-origin cells. Evidence from several laboratories has implicated it in carcinogenesis and tumor promotion [34].

Expression of TGF-β_1 in hamster cheek pouch carcinogenesis was studied immunohistochemically using two polyclonal antibodies directed against a synthetic polypeptide derived from the mouse TGF-β_1 sequence (gift of Drs. Sporn and Flanders). One antibody stains extracellular TGF-β_1 (CC) while with the other (CL) stains intracellularly [35]. Both antibodies cross-react efficiently in hamster tissues fixed in acetone.

In the normal cheek pouch, the expression of TGF-β_1 (CL) was restricted to differentiated cells but following a patched pattern throughout the length of the epithelium [36]. The reaction became more prominent and uniform (although still restricted to differentiated cells) in hyperplastic epithelia after multiple DMBA applications. In dysplastic hyperplasias, carcinomas *in situ*, and fully developed carcinomas, the reaction was negative. Reaction was also negative in verrucoid lesions.

The expression of the extracellular anti-TGF-β_1 antibody (CC) was restricted to the stroma near normal and hyperplastic epithelium. The reaction was particularly intense in areas adjacent to hyperplastic epithelium. The extracellular antibody was also positive in the stromal areas associated with preneoplastic and neoplastic tissues.

We have isolated RNA from normal and DMBA-treated cheek pouches as well as SCC in order to study the transcription of the TGF-β_1 gene. Preliminary results from a group of 5 carcinomas showed that the level of TGF-β_1 expression in these tumors is higher than in the untreated pouch. Increased transcription levels may be related to the presence of high levels of immunoreactive TGF-β_1 in the stroma of tumors and premalignant lesions [36].

These results suggest that loss of the inhibitory effect of TGF-β_1 may be one of the determinant changes of tumor development in this model. The possible function of extracellular TGF-β_1 in preneoplastic and neoplastic tissues remains to be determined, but it is likely that it may play a role in vascularization and permissive changes in the stroma for tumor invasion.

EXPRESSION OF CANCER-RELATED GENES

The level of expression of several cellular proto-oncogenes was examined at different stages of DMBA-induced tumor development in the hamster cheek pouch model [37]. Those studies demonstrated overexpression of the c-Ha-*ras* gene at a very early stage of tumor development. Conversely, expression of c-*erb*B was detected after 8–10 weeks of DMBA treatment and increased with the progression of the disease. Recent studies have also indicated that the c-*erb*B proto-oncogene and TGF-α may be involved in the process of chemical carcinogenesis in this system. The c-*erb*B gene, which is the cellular gene coding for EGF-R, has been found to be overexpressed in DMBA-treated pouch epithelium and in cheek pouch tumors. Furthermore, this gene appears to be amplified in cell lines derived from SCC [31]. Expression of c-*myc* and c-*sis* was detected at control levels, while c-*fos* gene activity could not be detected at any stage of tumor development. It has been suggested that increased expression of the *ras* gene can be correlated with the initial transformation of hamster cheek pouch epithelial cells, whereas activation of the c-*erb*B gene can be correlated with the extensive proliferative as

well as malignant phenotype of these cells in the intact animal [31].

GENETIC ALTERATIONS

The presence of an activating mutation in the Ha-*ras* gene in hamster cheek pouch tumors induced by repeated DMBA exposure was demonstrated by our laboratory. 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 hamster fragment was determined by direct sequencing 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 hamster cheek pouch SCCs were used to detect mutated Ha-*ras* alleles. DNA sequencing of the tumors showed that 6 of 11 tumors had an A to T transversion in the second position of codon 61, resulting in amino acid change from glycine to leucine. We also found that 3 of 7 tumors induced by the two-stage protocol (DMBA-BzPo) had the same mutation in codon 61 of the Ha-*ras* gene. As demonstrated in other systems, we have shown a specific mutation of the Ha-*ras* gene in chemically induced tumors of the hamster cheek pouch, further supporting the role of this oncogene in chemical carcinogenesis [38]. Recently, Bianchi *et al.* [39] postulated a possible cooperation between an activated *ras* gene and inactivation of a suppressor gene for the progression to malignancy in the mouse skin carcinogenesis model, raising the possibility of the existence of the same mechanism in the hamster cheek pouch model. Moroco *et al.* [40] have postulated the existence of three suppressor functions in this model, strongly suggesting the inactivation of suppressor genes.

Preliminary results from our laboratory also found accumulation of p53 protein in 20% of the DMBA-induced tumors of the cheek pouch with the complete carcinogenesis protocol. This suggests that p53 mutations are likely to play a role in this model. Ongoing studies are determining the position and nature of the mutation using the single strand conformational polymorphism technique and direct sequencing.

CONCLUSION

Studies in several laboratories have been focused on the development of reliable animal models that mimic critical events of human carcinogenesis. In this regard, the identification of biomarkers in the hamster cheek pouch carcinogenesis model and demonstration of their modulation represent important goals in the study of oral cancer. Histochemical and immunohistochemical studies have focused on a series of markers (*i.e.*, GGT, keratins) as well as alterations of regulatory proteins (TGF-β_1, EGF-R, ODC), which may reflect genetic changes in this system. We expect a detailed analysis of these markers to be useful in defining the different stages of tumor development and pathways of carcinogenesis. Along with these studies at the protein level, we have also obtained preliminary evidence which suggests genetic alterations, as well as altered expression of relevant genes at the RNA level in the hamster cheek pouch carcinogenesis model. We suggest that a number of these important biological and molecular markers could be excellent intermediate endpoints in assessing the effects of various chemopreventive agents in the hamster cheek pouch model and in human clinical trials.

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