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An experimental model to demonstrate the carcinogenic action of oral chronic traumatic ulcer

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BACKGROUND: Oral chronic traumatic ulcer (CTU) is caused by constant irritation by dental edges or restorations and could behave as a pre-malignant lesion in a field initiated by carcinogens such as tobacco or alcohol.
METHODS: We developed an experimental model in the hamster cheek pouch, combining the chemical carcinogen 7,12-dimethyl-benzanthracene (DMBA) with CTU.
RESULTS: The successive or simultaneous action of both agents induced a significantly larger number of endophytic carcinomas than larger doses of DMBA alone. CTU alone failed to induce tumor development. Ploidy analysis revealed significantly higher malignancy indices in endophytic than in exophytic carcinomas. 5-Bromo-2-deoxyuridine labeling evidenced greater proliferation around the ulcers in chemically cancerized epithelium than around ulcers in healthy epithelium.
CONCLUSIONS: The results show that CTU acts as a tumor promoter in this model. This finding is clinically relevant in that CTU may increase the risk of malignant transformation in patients with subclinical tumor initiation.
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Keywords: cell proliferation; experimental oral cancer; oral chronic traumatic ulcer; ploidy analysis; tumor promotion

Introduction

Chronic traumatic ulcer (CTU) of the oral cavity is a relatively frequent lesion that usually occurs in the posterior or middle third of the edge of the tongue or in the posterior third of the occlusal axis of the cheek mucosa. It is caused by constant, mild traumatisms inflicted by carious or fractured teeth or maladapted restorations or prosthesis. It has been suggested that if CTU is not treated adequately it may behave as a pre-cancerous lesion. This hypothesis is based on the existence of squamous cell carcinomas (SCC) flanking CTU of lengthy evolution and the fact that both lesions are more frequent on the edge of the tongue (1). However, there are no epidemiologic or experimental data that support this theory. The association between persistent inflammation as that caused by CTU and carcinogenesis has been postulated for different clinical conditions such as ulcerative chronic colitis and cancer of the colon (2), gastric ulcer and cancer (3, 4), chronic asbestosis, and lung mesothelioma (5). In all of these cases, the common factor is an increase in proliferation induced by inflammatory factors.

The role in the malignant transformation of squamous epithelia of agents that induce hyperplasia has been unequivocally determined in studies of experimental carcinogenesis in skin (6–8). These studies show that neoplasias develop as a result of a two-phase process: initiation and promotion. The initiating carcinogens are genotoxic compounds that, in small doses, produce changes in DNA that are not expressed as alterations in cell or tissue architecture. Promoter agents induce the proliferation of initiated cells. Most of the promoter agents that have been experimentally assessed are not carcinogenic *per se* and, in the absence of initiation, only induce reversible, hyperplastic changes.

Within this context, if oral mucosa were previously initiated by one of the most well-known carcinogens, i.e. tobacco and alcohol, we suggest a promoter action by the granulation tissue of CTU. This granulation tissue induces epithelial proliferation. However healing is prevented by constant irritation. Carcinogens such as tobacco and alcohol act on large areas of the mouth causing the phenomenon known as field cancerization (9).

We have developed a model of CTU by modifying the oral cancer model of most widespread use, the chemical cancerization of the hamster cheek pouch, simulating the constant irritation of the human CTU. As described in studies by other authors and by our laboratory, the model is highly reproducible (10–14) and produces two types of tumors at foreseeable times: highly differentiated exophytic carcinomas that resemble oral verrucous

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carcinomas and, at later times, less differentiated, more aggressive endophytic carcinomas that resemble SCC.

The interaction between the healing process of CTU and chemical carcinogenesis was analyzed in terms of the tumor type and yield, of ploidy-related parameters to objectively determine the degree of malignancy of the neoplasias, and of modifications of the epithelial proliferation index in the first stages of malignant transformation.

Materials and methods

Animals

We employed adult, male, and female Syrian Golden hamsters (*Mesocricetus auratus*), 6 weeks to 3 months of age, 100–150 g body weight. All the regulations of the National Institute of Health (1985) for the use and care of laboratory animals were observed.

Experimental protocols

We employed four experimental protocols:

Classical model of chemical cancerization (DMBA3)

We used the model described by Salley in 1954 (15) and standardized by Morris in 1961 (16). It involves topical application of 0.1 ml of 0.5% 7,12-dimethyl-benzanthracene (DMBA) in mineral oil in the right pouch of each animal three times a week.

The animals were killed at 19 weeks of treatment. In keeping with the literature and our own studies, exophytic and endophytic tumors develop at that time.

Experimental model of chronic traumatic ulcer (CTU)

The procedure involves causing a wound in the wall of the pouch under anesthesia. The pouch is everted and a wound with loss of substance, 8–10 mm in diameter, is inflicted to reach the loose adventitious tissue. As from the third day of wound-healing we scraped the surface of the wound with a scalpel eliminating the pseudomembrane to prevent epithelialization three times a week under anesthesia. In one group of animals this procedure was carried out over a period of 19 weeks whereas the group to be used as control in the study of initial effects on proliferation was treated for 4 weeks.

Model of cancerization by chemical initiation and traumatic promotion (DMBA3, CTU)

We carried out chemical cancerization with DMBA as previously described for the classical model but for 3 weeks only to allow solely for initiation (17, 18). Three days after the last dose of carcinogen, we inflicted a wound as above and applied the irritation procedure three times a week during the period of tumor promotion to complete the experimental period of 19 weeks. Another group of animals was killed at 7 weeks of treatment to evaluate initial effects on proliferation.

The group of control animals was submitted to topical application of 0.5% DMBA three times a week for 3 weeks and to no further treatment during identical experimental period to that employed for the other animals.

Model of simultaneous action of a chemical carcinogen and trauma (DMBA3, DMBA1+CTU)

We carried out chemical initiation over a period of 3 weeks (three times a week) and then inflicted a wound as above. During the period of tumor promotion, we irritated the wound on 2 of the 3 days of treatment and on the third we applied a dose of DMBA.

The control group was treated with DMBA three times a week for the first 3 weeks, and with DMBA only once a week during the promotion period.

Figure 1 schematically outlines the different experimental protocols and the experimental time-points examined.

Processing for histologic analysis

We injected the animals 30 min prior to kill intraperitoneally with 2 ml of a solution of 1% 5-bromo-2-deoxyuridine (BrdU), in keeping with the method of Sugihara et al. (19). The hamsters were killed by sulfuric ether inhalation, and the treated pouch of each animal was removed and fixed in 10% formaline. Following fixation the pouches were cut transversally to obtain specimens containing all the macroscopically visible lesions. The samples were routine processed, embedded in paraffin, sectioned and stained with hematoxylin and eosin. Adjacent sections were mounted for immunohistochemistry and ploidy analysis.

Immunohistochemical evaluation of proliferating cells

Following antigen retrieval with 0.1 M citrate buffer, pH 6, in a microwave oven, we performed BrdU detection using a mouse monoclonal anti-BrdU monoclonal antibody (clon IIB5; Biogenex, San Francisco, CA, USA)

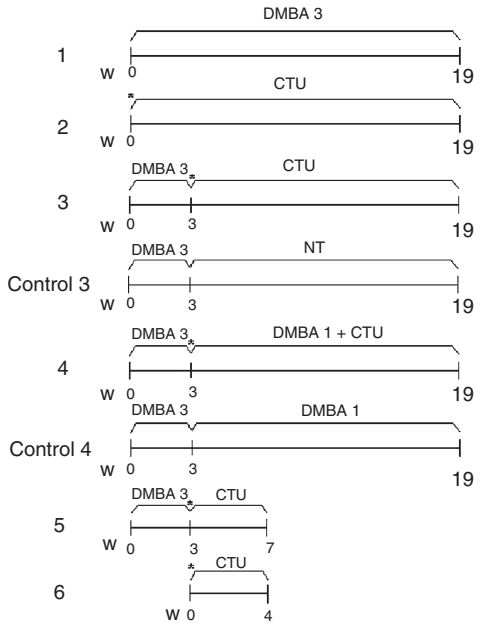

Figure 1 Experimental protocols. w, week of the experimental period; *wound; DMBA3, 7,12-dimethyl-benzanthracene (DMBA) 0.5% three times/week; DMBA1, DMBA 0.5% once a week; chronic traumatic ulcer (CTU), irritation of the surface of the ulcer, three times/week; DMBA1 + CTU, irritation of the surface of the ulcer twice a week + DMBA 0.5%, once a week; NT, no treatment.

and the biotin-streptavidine-peroxidase kit (Kit Multilink; Biogenex). The slides were counterstained with hematoxylin and mounted. We assessed a total of 15 animals submitted to CTU for a 4-week period: eight exposed to CTU only and seven that were initiated with DMBA3 (3 weeks) and promoted with CTU (experiments 5 and 6 of Fig. 1). We counted the labeled nuclei in areas of epithelium corresponding to 1000 μm of basal membrane length in both directions beginning at the edge of the wound. In the contralateral pouch wall, contained in the same section, we assessed an equal length of the epithelium to evaluate unwounded areas of epithelium untreated and treated with DMBA respectively.

Ploidy analysis

We performed the Feulgen reaction with hydrolysis in 5 N HCl for 90 min and incubation in Schiff reagent during 2 h (20). We undertook ploidy analysis by image analysis employing a Zeiss MPM 80 (Carl Zeiss, Jena, Germany) microscope and an IBAS-Kontron Electronic image analyzer with a Hitachi DK 7700-SX-K CCD (Hitachi Denshi Ltd., Tokyo, Japan) and DNA-IBAS software. Two correction factors were employed to improve the sensitivity of the method (21). The software affords the values of total optical density (TOD) of each nucleus, equivalent to the value of DNA content. In addition, it affords the mean ploidy value which results from the mean ratio of TOD and the TOD value of the lymphocytes in the same section taken as the 2c diploid control value, the ploidy histogram and the aggressiveness indices of Böcking (22). Briefly, these indices comprise:

1 Index of deviation from the diploid value (2cDI), which is defined as the sum of squares of the difference between the DNA values of single cells (c_i) and the 2c value, divided by the number of measured cells (N):

$$2\text{cDI} = \frac{1}{N}\sum_{i=1}^{N}(c_i - 2c)^2$$

2cDI therefore represents the mean square deviation from the diploid value.

2 5c exceeding rate (5cER), which is defined as the percentage of cells with a DNA content of more than 5c. 5cER therefore indicates the degree of aneuploidy.
3 Malignancy index (MI), which is the product of 2cDI and 5cER:

$$\frac{2\text{cDI} \times 5\text{cER}}{100}$$

We evaluated eight exophytic tumors and five endophytic tumors.

Results

Macro- and microscopic findings

In the classical cancerization protocol with DMBA the exophytic tumors began to appear at 10–12 weeks after the start of the experiment as small masses (1–2 mm in diameter) that reached diameters of 1–1.5 cm at kill (19 weeks). The endophytic tumors were less abundant and appeared as hard masses in the thickness of the pouch wall at 16 weeks of treatment.

Histologically, exophytic tumors are highly keratinized verrucous carcinomas that exhibit varying degrees of cell atypia. The endophytic tumors are composed of thinner epithelial cords that infiltrate the thickness of the pouch wall, are less differentiated and have more cell atypia than exophytic tumors.

The wounds maintained in pouches that were not submitted to treatment with DMBA exhibited a bed composed of granulation tissue with abundant lymphoplasmocytic infiltrate. The epithelial edges were acanthotic and torn at the ends as a result of the mechanical trauma. They merged with a haemorrhagic surface with polymorphonuclear neutrophils. The histologic features were similar for all the experimental time-points examined (3, 7, and 19 weeks).

The sequential or simultaneous association between chemical cancerization and traumatic ulcer, induced neoplastic proliferation on the edges of the wound (Fig. 2). The acanthotic epithelium gave rise to infiltrating cords made up of atypic cells (Fig. 3) that in some cases gave rise to large tumor masses that filled the ulcer bed (Fig. 4). Exo-endophytic tumors were occasionally observed. Endophytic and exophytic tumors developed in other areas of the pouch. It is noteworthy that in this case the number of DMBA doses required to induce tumor development was much less that in the classical experiment.

Table 1 shows the incidence of tumors in experimental and control animals and the dose of DMBA in each case. CTU alone failed to induce tumors. The incidence of exophytic tumors varied with the dose of DMBA administered. Nine and 25 doses of DMBA failed to induce endophytic tumors. When chronic trauma is coupled to these doses, endophytic tumors that resemble human SCC appear in significantly higher proportions than when tumors are induced by 57 doses of DMBA.

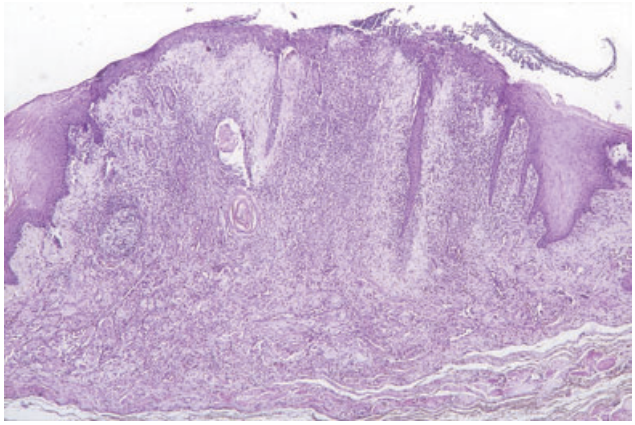

Figure 2 Traumatic ulcer induced in an epithelium previously cancerized over a 3-week period and maintained for a subsequent period of 4 weeks. Epithelial cords can be seen infiltrating the granulation tissue of the wound bed (×100).

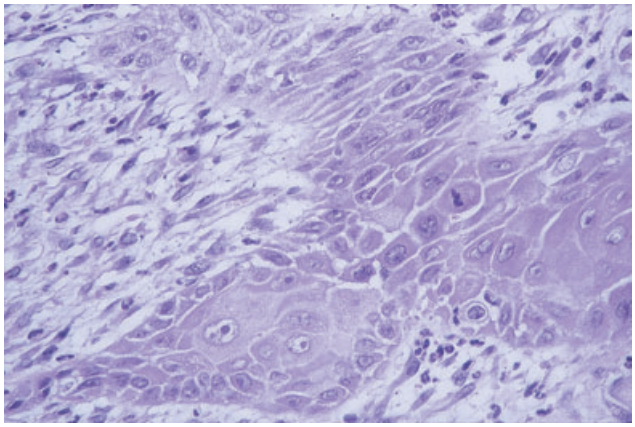

Figure 3 Tumoral cord exhibiting polymorphism and mitosis can be seen infiltrating wound-healing tissue (×400).

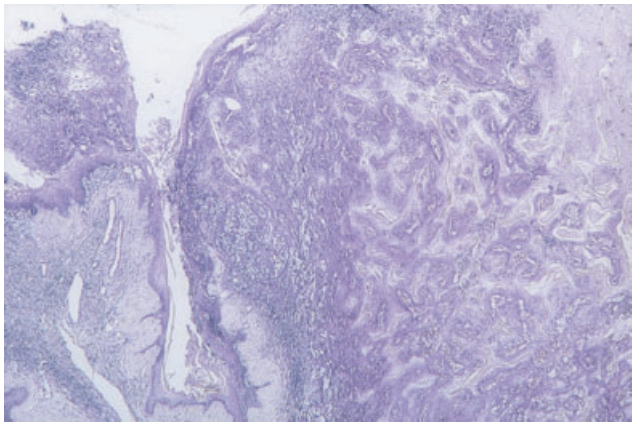

Figure 4 Endophytic carcinoma can be seen infiltrating the ulcer bed. Nineteen weeks of cancerization and maintenance of the ulcer (×50).

Ploidy analysis

Figure 5 shows an example of a histogram for endophytic and exophytic tumors. A more marked skew to the right from the diploid value was observed in all endophytic tumors. The frequency peak of all exophytic tumors was in the diploid–tetraploid range, whereas four of five cases of endophytic tumors defined one or two peaks in the tetraploid-aneuploid regions. The values of mean ploidy and of the indices of deviations from 2c, aneuploidy and malignancy were significantly higher in endophytic than in exophytic carcinomas (Table 2). These findings indicate that these two tumor types differ markedly in terms of aggressiveness.

Proliferative activity

Proliferative activity was assessed at 7 weeks of treatment when no tumors have developed yet. The normal epithelium that was not treated with DMBA exhibits, approximately, one labeled nucleus per 200 μm of basal membrane. Quantitative evaluation of labeled nuclei afforded a mean value of labeling index (LI; i.e. number of labeled nuclei per 100 μm of basal membrane) of 0.40 (Fig. 6). Topical application of DMBA during 3 weeks elicits a rise in LI to 0.45. However, this difference did not reach statistical significance. The epithelial edges of the wounds inflicted in untreated normal epithelia and maintained with irritation for 4 weeks showed a LI of 1.41, whereas similar ulcerations produced in epithelium previously initiated with DMBA featured a LI of 3.79.

Discussion

Wound-healing and carcinogenesis have certain biologic characteristics in common. It has been shown that many chemical mediators released by healing tissue induce neovascularization, inflammation, cell proliferation, synthesis of collagen and other substances of the extracellular matrix, all of which are involved not only in wound-healing but also in fetal growth and malignant transformation of tissues (23–25).

However, the main difference lies in the proliferation of transformed cells. In wounds, cells proliferate to allow for healing and cease to do so when healing has been completed. This process is regulated by the interaction between the cells that make up the newly formed structures. In malignant tumors, cell proliferation is deregulated as a result of faulty cell cycle regulation caused in turn by abnormal proteins encoded by activated oncogenes. The remaining factors, encoded by normal genes, act as in wound-healing processes and contribute significantly to tumor growth, vascularization and formation of supporting stroma. This fact explains the tumor promoter effect of agents that induce epithelial hyperplasia and concomitantly induce inflammation, and require prior initiation of the epithelium to induce tumor

Table 1 Incidence of tumors after 19 weeks of treatment

Treatment	*Total number of animals*	*Number of DMBA doses*	*Number of animals with tumors*	*Percentage of animals with exophytic tumors*	*Percentage of animals with exo-endophytic tumors*
1. DMBA3	28	57	24	87.5 (21)	12.5 (3)
2. CTU	19	0	0	0 (0)	0 (0)
3. DMBA3, CTU	19	9	6	16.67 (1)	83.33 (5)*
Control 3	12	9	0	0 (0)	0 (0)
4. DMBA3, DMBA1 + CTU	18	25	18	38.88 (7)	61.11 (11)*
Control 4	14	25	8	57.14 (8)	0 (0)

The number of animals in each category is indicated in brackets.
*Statistically significant differences between each group and the DMBA3 group ($P < 0.05$) – chi-square test.
DMBA, 7,12-dimethyl-benzanthracene; CTU, chronic traumatic ulcer.

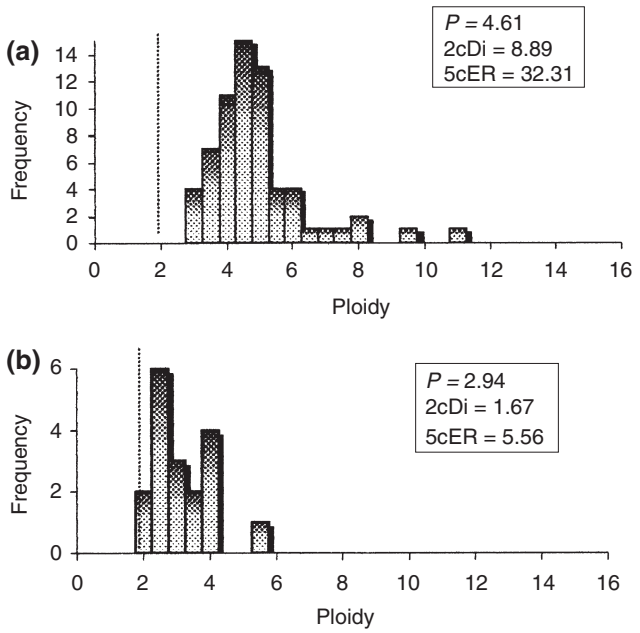

Figure 5 Examples of DNA ploidy histograms for endophytic (a) and exophytic (b) tumors. Most cells in exophytic tumor fall within the 2c–4c range, with a peak in the diploid value whereas a skew to the right is observed in the endophytic tumor. A higher frequency of cells in the aneuploid region is also observed. Böcking's indexes also show a higher grade of malignancy in endophytic tumor. P, mean ploidy value; 2cDI, index of deviation from the diploid value; 5cER, aneuploidy index.

Table 2 Analysis of tumor ploidy

Index	*Exophytic tumors*	*Endophytic tumors*	*Student's t-test*
Ploidy	3.07 ± 0.44	5.10 ± 1.48	$P = 0.00018$
2cDI[a]	2.43 ± 1.27	15.41 ± 6.35	$P = 0.00636$
5cER[b]	8.35 ± 6.03	34.36 ± 8.27	$P = 0.000001$
MI[c]	0.25 ± 0.27	6.26 ± 8.40	$P = 0.011846$

We analyzed eight exophytic tumors and five endophytic tumors.
[a]Polyploidy, [b]Aneuploidy, [c]Malignancy [in keeping with Böcking et al. (22)].
2cDI, index of deviation from the diploid value; 5cER, 5c exceeding rate; MI, malignancy index.

formation. This fact, so clearly evidenced in epidermis (6–8), has also been demonstrated in oral mucosa employing the model of chemical cancerization in the hamster cheek pouch (17, 18). The process, known as two-stage carcinogenesis, was evidenced employing 0.5% DMBA for 3–4 weeks as an initiating agent and 12-*o*-tetradecanoylphorbol-13-acetate (TPA) as a promoter agent. We employed the same chemical initiation process. The DMBA3, CTU protocol allowed us to separate the two stages and provide evidence of the promoter action of the inflamed granulation tissue of CTU in the development of tumors. The protocol DMBA3, DMBA1 + CTU combines both effects but simulates more closely what would happen in the human mouth when CTU occurs in smoking or alcoholic patients.

Similarly to what occurs with alcohol or the combustion products of tobacco, when carcinogens

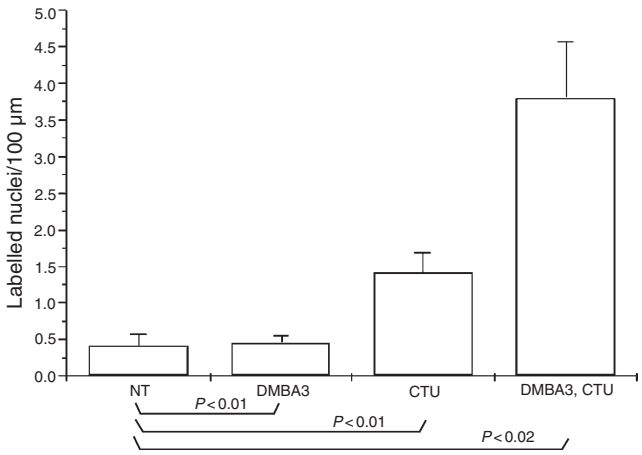

Figure 6 Number of 5-bromo-2-deoxyuridine (BrdU)-positive nuclei for the different experimental conditions. NT: no treatment; DMBA3: 7,12-dimethyl-benzanthracene (DMBA) three times/week for 3 weeks; chronic traumatic ulcer (CTU): edge of traumatic ulcer in healthy epithelium; DMBA3, CTU: edge of traumatic ulcer in previously initiated epithelium.

come into contact with the hamster cheek pouch, they spread over the whole surface of the mucosa giving rise to what is known as 'field cancerization' (9). Thereafter, exophytic tumors develop at different sites of the mucosa. These exophytic tumors were initially erroneously classified as papillomas. They are constituted by invaginated and hyperkeratinized epithelium but do have cell atypia and are highly proliferative. Endophytic tumors appear at a later stage, are infiltrating and less differentiated and closely resemble human SCC. Aside from the routine histologic characterization and the difference in DMBA doses necessary to induce the different tumor types, the biologic differences between exophytic and endophytic tumors have been studied only to a small extent. Giménez-Conti et al. (26) showed an increase in the accumulation of the protein p53 in endophytic tumors. The present study contributes the data of the ploidy study. DNA measurement performed by image analysis is now a widely used method to grade malignancy in solid human tumors with diagnostic or prognostic purposes (27). The method has also been used to characterize different experimental animal models of cancer (28–30). However, extrapolation of results to human oncology requires a cautious interpretation. The potent genotoxic carcinogen employed to induce tumors in the hamster cheek pouch model described herein leads to the formation of carcinomas of a higher grade of malignancy than that generally observed for oral human tumors. In the present work, results of ploidy analysis correlate with histologic observations, and were used only as an objective tool to characterize the different grades of malignancy already subjectively described for exophytic and endophytic tumors.

In the model employed herein, repeated irritation of the surface of the ulcer prevents extension of the epithelial projection that arises on the acanthotic edges of the wound to cover the granulation tissue. In this way, it prevents it from maturing to fibrosis, and the inflamma-

tory granulation persists. The data obtained employing BrdU labeling as the end-point suggest that when these effects occur in an epithelium with genomic changes that deregulate the cell cycle, the same proliferative stimuli lead to an increase in the mitosis of altered cells that in turn give rise to infiltrating, neoplastic growth.

The present data are directly relevant to clinical oral medicine within the context that CTUs do not, as such, increase the risk of malignant transformation but could act as a promoter agent in smoking or alcoholic patients with subclinical alterations that characterize tumor initiation.

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