EARLY PHENOTYPIC AND GENOTYPIC ALTERATIONS IN SUBMANDIBULAR GLAND ONCOGENESIS IN RATS

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ABSTRACT

The present study evaluates the phenotypic and genotypic changes that take place during early oncogenesis. The submandibular glands of male rats were injected with a 0.5% solution of 9,10-dimethyl-1,2-benzanthracene (DMBA) in acetone. Gland samples were taken at 0, 7, 30 and 150 days post-injection and submitted to histological, biochemical, immunocytochemical and PCR evaluation. Histopathological analysis was performed on hematoxylin-eosin stained slides. Total protein content was assessed by Lowry's method and the protein profile was analyzed by 12% SDS-PAGE. Bcl-2 was demonstrated by silver-enhanced gold immunolabeling. p53 immunolabeling was performed using the streptavidin-biotin system. All the treated animals developed carcinoma-like lesions at 30 and 150 days. Total protein concentration rose significantly ($p<0.05$) above control values at 7, 30 and 150 days. The treated glands exhibited positive immunolabeling for p53 in the nuclei of neoplastic cells at 30 and 150 days. Treated glands also showed positive cytoplasmic immunolabeling for Bcl-2, exhibiting statistically significant differences between 7, 30 and 150 days ($p=0.0015$), and with controls ($p<0.0001$). No p53 mutations were observed whereas a point mutation, C-to-A, of the Bcl-2 gene was detected at 7, 30 and 150 days by PCR amplification. This mutation led to a single aminoacid change (thre→ asn) in the protein molecule. Our results suggest that the early histopathological changes correspond to quantitative and qualitative protein changes. The histopathological, biochemical, immunocytochemical and genetic alterations observed during the course of experimental carcinogenesis in the submandibular gland of the rat could constitute reproducible indices of malignant transformation applicable to human oncogenesis, given the high degree of homology between the oncogenes of mice, rats and human beings.

***Key words**: rat submandibular gland, DMBA, experimental oncogenesis, p53, Bcl-2.*

ALTERACIONES TEMPRANAS FENOTÍPICAS Y GENOTÍPICAS EN LA ONCOGÉNESIS DE GLÁNDULA SUBMANDIBULAR DE RATA

RESUMEN

Se evaluaron modificaciones del fenotipo y genotipo en glándulas submandibulares durante el desarrollo temprano de la tumorogénesis. Glándulas submandibulares de ratas macho fueron inyectadas con una solución al 0,5% de 9,10-dimetyl 1,2-benzanthracene (DMBA), diluida en acetona. Muestras de glándulas fueron analizadas mediante técnicas histológicas, bioquímicas, imunocitoquímicas y por PCR a los 0, 7, 30 y 150 post inyección. Para los estudios histopatológicos se utilizó la técnica de Hematoxilina- Eosina. Se determinó la concentración de proteínas totales por el método de Lowry y se realizaron corridas electroforéticas en gel de poliacrilamida SDS-PAGE al 12% para determinar el perfil proteico. Se realizó inmunomarcación para Bcl-2 con oro coloidal-plata y para p53 por streptavidina-biotina. Todos los animales tratados desarrollaron cambios similares a carcinomas a los 30 y 150 días. La concentración de proteínas totales aumentó significativamente ($p <0,05$) a los 7, 30 y 150 días en relación a los controles. En glándulas inducidas la inmunomarcación fue positiva para la proteína p53 en núcleos de células neoplásicas a los 30 y 150 días. En las mismas glándulas, la marcación citoplasmática de Bcl-2 fue positiva a los 7, 30 y 150 días ($p=0,0015$) y en relación a los controles ($p<0,0001$). No se observaron mutaciones de p53 mientras que se observó una mutación puntual, C→ A, del gen bcl-2 a los 7, 30 y 150 días que generó un cambio de aminoácidos en la proteína (thre→ asn). Nuestros resultados sugieren que los cambios histopatológicos tempranos corresponden a modificaciones cuantitativas y cualitativas de las proteínas. Las modificaciones observadas a nivel histopatológico, bioquímico, inmunocitoquímico y genético en la carcinogénesis experimental de glándula submandibular de rata podrían representar parámetros reproducibles de transformaciones malignas transferibles al ser humano, dada la alta homología de estos oncogenes entre rata, ratones y el hombre.

***Palabras clave**: glándula submandibular de rata, DMBA, oncogénesis experimental, p53, Bcl-2.*

INTRODUCTION

Salivary gland tumors are a heterogeneous group of neoplasms. The study of their pathogeny has been complicated by this heterogeneity. The incidence of these tumors is 2.5-3.0 cases per 100.000 people per year in the western world. Approximately 80% are benign lesions and 0.5% exhibit malignant features (1). Of the three major salivary glands, the submandibular gland is the site that exhibits the worst prognosis (2). Patients with submandibular gland tumors that have been subjected to surgical excision of the glands exhibit clinical reduction in salivary flow, alterations in the cariogenic flora, a rise in dental caries and psychological effects (2).

The process that leads to the malignant transformation of these tumors is not fully understood. Thus, tumor classification is based essentially on morphological and histological features (2). However, in the development of salivary gland hybrid carcinomas, genetic-molecular events are thought to be pivotal in the transformation of a low-grade malignancy tumor to a high-grade tumor (3). Tumor progression is characterized by cell proliferation and apoptosis. Both of these processes are regulated by a group of tumor suppressor oncogenes and genes (4). Amongst them, of particular relevance are p53, present in 50% of all studied human cancers (5), and Bcl-2, frequently associated to degree of malignancy in human cancer (6,7).

The wild type p53 protein is a powerful inhibitor of the cell cycle and controls both cell growth and the apoptosis of cells with DNA damage induced by environmental factors. Molecular biology studies of salivary gland adenocystic carcinomas evidenced selective genetic mutations of p53 during the process of dedifferentiation (8). Malignant myoepithelioma, a rare salivary gland tumor, exhibited overexpression of p53, marked pleomorphism and high cell proliferation (9). The structural mutation of the wild type p53 inactivates the protein, impairing its function. This process would contribute to tumor malignancy (7,10). In the eighties, immunolabeling studies showed that the p53 protein localizes preferentially in the cytoplasm in most normal cells. However, p53-positive immunolabeling increases in the nucleus of transformed or highly proliferative cells (11). The p53 protein is associated to the Mdm2 protein, a negative regulator of the nucleus-cytoplasm transport of p53. The high levels of p53 in the nucleus generally indicate pathology related to DNA alterations. However, potential changes in cytoplasmic factors related to the inhibition of return transport to the nucleus may be involved (11).

The Bcl-2 oncoprotein is involved in the process of apoptosis and is increased in mammary, colorectal, lung, prostate, thyroid, bladder, ovary, head and neck neoplasms and melanoma (12). The Bcl-2 family (Bax, Bad, Bid, Bak, Bcl-XS, Bik, Bim and HRK) (13) may play a dual role by permeabilizing the external membrane of the mitochondria, allowing the exit of cytochrome c and stimulating apoptosis and, conversely, inhibiting apoptosis by impairing the exit of cytochrome c (14). The antiapoptotic function of this family prevails in tumorigenesis.

A late diagnosis of already established human salivary gland tumors might imply irreversible damage and bad prognosis. The deficient therapeutic approach is generally the result of the poor understanding of the process of malignant transformation in salivary glands due to the impossibility of obtaining biopsies during the early stages of tumorigenesis in contrast to other tumors of the oral cavity (1,15,16). Within this context, experimental models are essential to study the early stages of malignant transformation in salivary glands. These models mimic the early patterns of molecular and cellular change (17,18). The carcinogen DMBA has been widely used to induce tumors in hamster oral mucosa (19,20) and in the submandibular glands of rats and mice (17,18,21). In general, gland models are used to study long-term events that occur when the tumor is already established (18). Very few models, and particularly very few rat models, have been used to study the early genotypic and phenotypic changes that take place from the onset of DMBA induction. Undoubtedly, the characterization of molecular and cellular patterns could be used jointly with the clinical characterization to evaluate sensitivity and degree of malignancy in preneoplastic and neoplastic lesions.

The aim of the present study was to evaluate the early phenotypic and genotypic changes in oncoproteins p53 and Bcl-2 in an experimental model of DMBA-induced tumorigenesis in submandibular gland. The findings will serve to determine a pattern of early tumorigenic changes and characterize a model that can be employed in studies on salivary gland cancer that are relevant to the prevention and therapy of the disease.

MATERIALS AND METHODS

The protocol for the use of experimental animals was designed in strict observance of NIH guidelines. The protocols were reviewed and approved by the Research Ethics Committee of the "Niño Jesús" Children's Hospital of the Province of Córdoba.

Male, 60 day old Wistar rats (n=50), approximately 180 g in body weight were used throughout. The Cell Biology Institute of the University of Córdoba provided the animals. The rats were fed a nutritionally balanced diet and allowed access to water *ad libitum,* kept in controlled temperature at 24ºC with 12:12 h light/dark cycles. The animals were divided up into 2 groups: a) experimental (n=25), given an injection of 50 µl of 0.5% of DMBA in each submandibular gland; b) control (n=25), given an injection of 50 µl of 0.9% physiological solution.

Tumor induction by DMBA

All the animals were anesthetized with a solution of 1.28 mg Xylazine / 8 mg Ketamine / 100 g body weight (Bayer, Argentina and Ketalar, Parker Davis, respectively). A small incision was made on the ventral surface of the neck and the subcutaneous tissue was set aside to expose both submandibular glands.

The exposed glands of the experimental group were injected with 50 µl of 0.5% of 9, 10- dimethyl 1, 2 benzanthracene (DMBA) (SIGMA ALDRICH, USA) dissolved in acetone. The glands of the control group were injected with a 0.9% sodium chloride solution. The incision was carefully sutured with suture thread.

The submandibular glands were excised at 0, 7, 30 and 150 days post-treatment, weighed on a precision balance and measured with a caliper. The weight was expressed in grams and the volume in mm^3 (17). In all cases the right gland was employed for light microscopy analysis and the left gland was used for biochemical, genetic and immunolabeling studies.

Histological Analysis

The glands were fixed in 10% formaline buffered with hydrochloric acid-acetyl pyridine pH 7, processed for paraffin embedding, sectioned and stained with hematoxylin-eosin (H-E). The sections were examined in an Olympus BX50 microscope and the histological images were digitalized with a SONY camera and Pro-plus software.

Biochemical analyses

Small portions of gland tissue were homogenized in 0.9% saline solution and centrifuged at 18,000 g. The total protein content was determined in the supernatant by Lowry's meted (22). Appropriate samples were analyzed by 12% SDS-PAGE at room temperature in keeping with Laemmli (23). 50 µg samples were loaded in each lane. The gels were stained with 0.1% Coomassie Blue R-250.

Immunolabeling

Immunocytochemistry was performed employing the streptavidin-biotin complex technique or colloidal gold immunolabeling. The sections were dewaxed in xylene, hydrated in decreasing ethanol concentrations and rinsed in distilled water. The sections were incubated overnight with IgG anti-p53 (DAKO, USA) and anti-Bcl-2 (DAKO, USA), 1:100 in PBS, in a damp chamber at 4ºC. The secondary anti-mouse antibody was diluted 1:50 in PBS. Bcl-2 immunolabeling was performed employing the colloidal gold technique (10 nm gold particles, Sigma-Aldrich, USA). The sections were incubated with the second antibody at room temperature for 2-3 hours (15) and immunolabeling was visualized by silver enhancement (BioCell, UK). Immunolabeling of p53 was evidenced by 3, 3'-diaminobenzidine (DAKO, USA). The sections were counterstained with Harris' hematoxylin and mounted in Entellan (Merck). Negative controls in which the primary antibody was withheld were run simultaneously.

p53-positive nuclei were counted at each experimental time and expressed in relation to the total number of nuclei in 50 mm^2. Gold-silver particles labeling Bcl-2 were evaluated and expressed in relation to the total number of cells in 50 mm^2. The values represent the median value of 3 independent samples of 8 sections each. The sections were evaluated by light microscopy at a magnification of 200x (15,24).

DNA isolation and amplification

Genomic DNA was isolated from 10-20 g samples of tumoral submandibular glands, employing the Wizard Genomic DNA purification kit (Promega). The $^{260}/_{280}$ absorbance ratio of the DNA employed for PCR was ≥ 1.70.

Mutations were detected employing the Polymerase Chain Reaction (PCR). The primers (Table 1) designed for p53 and Bcl-2 corresponded to the

TABLE I. Oligonucleotides (primers) designed and used for the amplification of the rat genes TP53 and Bcl-2.		
	Sequence of the primer	**pb**
p 53 sense	5'-TCATCTCCCTGCCAGATAGTCCACC-3'	736
p53 antisense	5'-GCTAAAGAGGAACCCCAAAATCTAGACAGAG-3'	
bcl-2 sense	5'-CGGGAGCCGGGACGCGAAGTG-3'	575
bcl-2 antisense	5'-GAGCATCCCCGCCTTGAGATCAAAGCC-3'	

sequences at positions 6891-7627 and 237-812 of the sequence of nucleotides of *Rattus norvegicus* published in GenBank, access numbers NW_047334 and NW_047390, respectively. The PCR reaction was done in a 50 µl final volume. PCR amplification was carried out on BioRad's iCycler thermal cycler according to the following protocol: 10 min at 95ºC, 1 min at 95ºC, 1min at 62ºC, and 2 min at 72ºC for 40 cycles, with an additional 10 min at 72ºC after the last cycle. The PCR products were separated on a 0.8% TBE agarose gel and stained with ethidium bromide. A DNA ladder marker (Promega, USA) was used to size the DNA fragments. The fragments were purified and sequenced at Macrogen Inc., Seoul, Korea.

Statistical analysis

The data of protein concentration (experimental vs. control) were analyzed with two-way ANOVA and *a posteriori* orthogonal contrasts employing the SPSS software, 10.1 version for Windows, 1999. Statistical significance was set at p=0.05. Protein concentration was considered a dependent variable whereas time and treatments were considered independent variables (25).

Immunolabeling data for p53 and Bcl-2 at the different experimental times were analyzed with a Generalized Linear Model (GLM) with nested design under Poisson distribution (26). Statistical significance was set at p=0.05. The scale parameter employed for the estimations was the square root of the Deviance employing PROC GENMOD (SAS software, version 8, 1999-2000). A binary variable was designed to analyze the difference in immunolabeling between the treated and control groups, i.e. positive immunolabeling: 1, negative immunolabeling: 0. Immunolabeling was considered negative when the number of nuclei or gold/silver particles/area was less than 10%. χ^2 was used to test the hypothesis of homogeneity of proportions (27).

RESULTS

All the animals whose submandibular glands were injected with DMBA developed tumors in the neck region. At 7, 20 and 30 days post-injection all the animals exhibited significant congestion in the region of the surgical incision. Hypertophic lymph nodes were observed around the glands. At 150 days post-injection, the glands of all the animals exhibited nodular masses with white-grey surfaces surrounded by fibrous tissue.

No macroscopic or histological tumoral changes were observed in control animals. Light microscopy analysis of the submandibular glands revealed the presence of basophilic acini devoid of secretory material in the lumen of striated and excretory ducts and convoluted granular tubules, in keeping with previous descriptions of normal glands (Fig. 1G).

All the histopathological descriptions were performed in keeping with the histological typing of the World Health Organization (WHO) (28).

At 7 days, the glands of the treated group of animals, exhibited epithelial, proliferative neoplastic cells invading the stroma as cords, nests or epithelial pearls (Fig. 1A). Structures resembling ducts, some with epidermoid differentiation, were observed at 30 days (Fig. 1B). Unequivocal tumor development was observed at 150 days. Some tumors exhibited nests of cells with microcysts containing basophilic secretion. Other tumors showed a disorganized parenchyma with tumor cell cords.

Total protein concentration was significantly higher in the DMBA-treated groups than in the control groups at all time points ($p<0.05$) (Fig. 2A). No interaction was observed between time and treat-

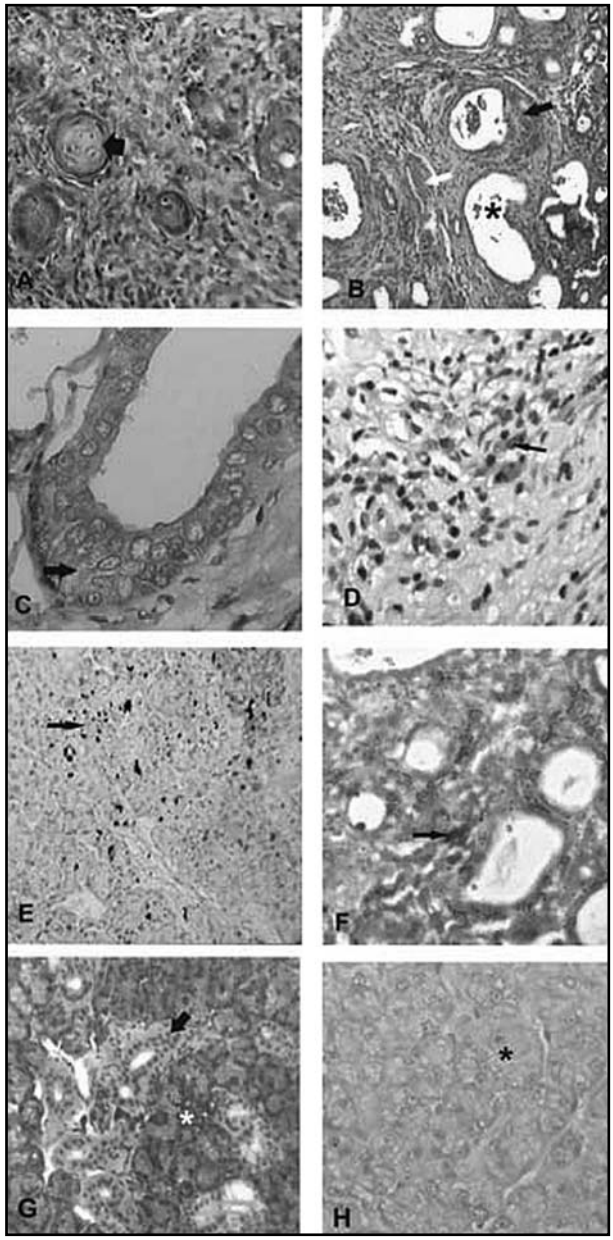

Fig. 1: *Histological and immunohistochemical findings in submandibular gland.* ***A:*** *7 days post-DMBA injection, Arrow: epithelial cells invading stroma, arranged in cords and nests and epithelial pearl formations (H-E stain x200).* ***B:*** *30 days post-DMBA injection. (*), duct-like structures, white arrow: hemorrhage, black arrow: fibrous tissue surrounding the tumor nests (H-E stain x200).* ***C:*** *p53 immunolabeling at 7 days post-DMBA injection. Arrow: positive cytoplasmic reaction in normal ducts (Harris hematoxylin stain x400).* ***D:*** *p53 immunolabeling at 30 days post-DMBA injection. Arrow: positive nuclei (Harris hematoxylin stain x400).* ***E:*** *Bcl-2 immunolabeling at 7 days post-DMBA injection. Arrow: gold-silver particles (Harris hematoxylin stain x200).* ***F:*** *Bcl-2 immunolabeling at 30 days post-DMBA injection. Arrow: gold-silver particles (Harris hematoxylin stain x400).* ***G:*** *control gland. (*), serous acini. Arrow: ducts. (H-E stain x400).* ***H:*** *immunolabeling in control gland. (*), acini (Harris hematoxylin stain x200).*

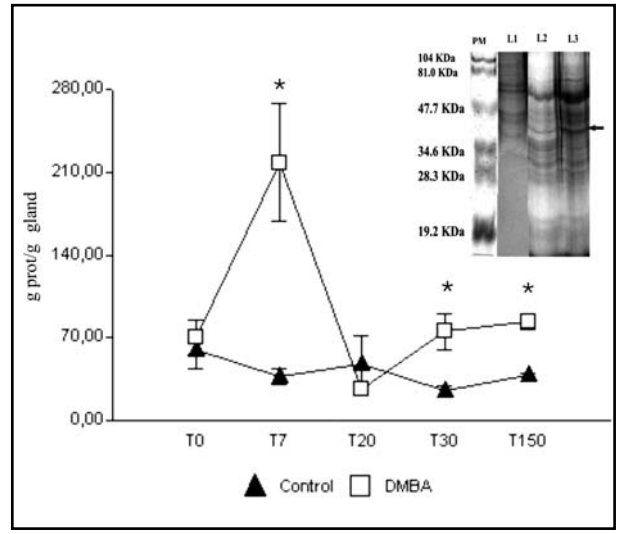

Fig. 2: A: *Total protein concentration (g prot/g gland) of submandibular gland at 0, 7, 20, 30 and 150 days post injection with 0.5% DMBA or 0.9% NaCl (controls). Values represent average of three independent experiments ± SE. (*): statistically significant differences (p<0.05).* ***B:*** *Protein pattern of submandibular gland by SDS–PAGE 12% stained with Coomasie Brilliant Blue R-250.* ***PM:*** *standard weight protein.* ***Lane 1:*** *control.* ***Lane 2:*** *DMBA-treated on day 7.* ***Lane 3:*** *DMBA-treated on day 30.*

ment (p<0.05). SDS-PAGE analysis of protein patterns revealed an increase in a 60 Kda band above control at 7 and 30 days and the presence of a new band at approximately 30-50 Kda (Fig. 2B).

At 7 days, DMBA-treated glands exhibited positive immunolabeling for p53 in the cytoplasm of normal duct cells (Fig. 1C). However, at 30 and 150 days, immunolabeling was significantly positive (p<0.0001) in the cell nucleus as compared to control (Fig. 1D).

At 7, 30 and 150 days post-injection of DMBA, cell cytoplasms exhibited significant positive Bcl-2 immunolabeling as compared to control (p<0.0001) (Fig. 1E-F). The increase in positive immunolabeling was also statistically significant at 7 and 30 days (p=0.0015) (Table 2).

Selected regions of TP53 and Bcl-2 genes were sequentiated for control and treated animals. No mutations were observed in the PCR products of control animals. Mutations were found in DMBA-treated animals, i.e. the mis-sense C→A mutation in the sense and antisense sequences of the Bcl-2 gene (Figure 3; Table 3). The mutation in the Bcl-2 gene resulted in an aminoacid change (threo→asn) (Table 3). No mutations were observed in the sequentiated exons of the TP53 gene.

TABLE II. Percentage of positive immunolabeling for p53 and Bcl-2 in control and DMBA-treated submandibular glands.

Time after treatment (days)	Control(**) p53	Bcl-2	DMBA (mean SD) p53	Bcl-2
7	0	0	0	13.4+ 0.34
30	0	0	11.3+ 0.14	32.4+ 0.46(*)
150	0	0	11.2+ 0.28	54.6+ 0.81 (*)

0 indicates negative immunolabeling and corresponds to values < 10 % of nuclei or gold-silver particles with positive labeling in each field evaluated. (*) Indicates statistically significant differences for the same marker between the time-points evaluated. ($p<0.05$); (**) Indicates statistically significant differences between the control and treatment groups at all the time-points evaluated ($p<0.05$).

TABLE III. Mutations and immunolabeling in animals treated with DMBA.

Gene	Time (days)	Base change	Aminoacid change	p53 Immunolabeling	bcl-2 Immunolabeling
p53	7	none	----------	(-)	(+)
	30	none	----------	(-)	(+)
	150	none	----------	(-)	(+)
bcl-2	7	ACC→AAC	Thre→asn	(-)	(+)
	30	ACC→AAC	Thre→asn	(-)	(+)
	150	ACC→AAC	Thre→asn	(-)	(+)

(-)Indicates immunolabeling <10%; (+) Indicates immunolabeling >10%

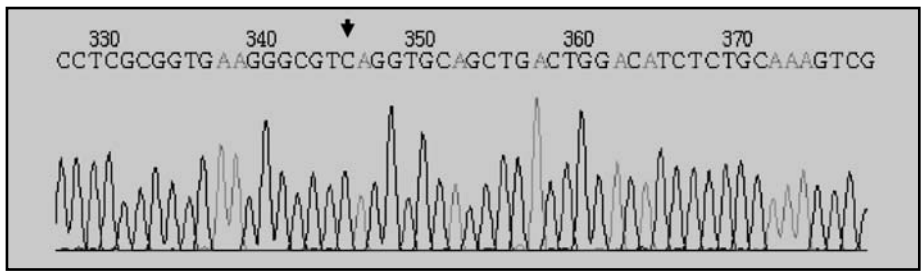

Fig. 3: *Sense sequencing of Bcl-2 of submandibular gland post injection with DMBA. Arrow indicates base change.*

DISCUSSION

Early histopathological diagnosis coupled to the detection of biochemical-genetic alterations would contribute to prevent further tumor development, improve prognosis and reduce the risk of the development of a neoplastic lesion. This would be particularly relevant in the case of tumors of the submandibular gland that develop into highly malignant lesions (28,29).

Research on carcinogenesis of submandibular glands revealed that DMBA induces the formation of different tumor types (18,20,21). This differential response would be associated to the way in which each tissue responds to carcinogens and the multicausality of tumor formation (18). Within this context, the characterization of the different animal models is essential to standardize responses and obtain comparable data that can be extrapolated to human pathology.

The present study reveals the presence of histological changes in 40-50% of the gland structure at 7

days post-treatment with DMBA These alterations could correspond to early changes associated to tumorigenesis because they resemble the features of a low-grade squamous cell carcinoma (20,28). At later times we observed disorganization of the parenchyma with proliferation of neoplastic cells to give rise to duct-like structures (18,21,28).

As from day 7 post-treatment with DMBA, the area of the surgical incision exhibited marked congestion compatible with angiogenesis. The growth of solid tumors is strictly dependent on the formation of a vascular network originated in the blood vessels of the host. Previous studies on different human tumor types with profusely microvascularized areas revealed a strong association between tumor development and progression (31) and the vascular endothelial growth factor (VEGF). Furthermore, VEGF increases cell survival by activation of the expression of the Bcl-2 gene (32). This finding coincides with the positive Bcl-2 immunolabeling, the genetic mutations in Bcl-2 and the corresponding genetic sequence as from day 7 post-injection with DMBA described herein (Table 3).

At a biochemical level, at 7 and 30 days post-injection of DMBA, the electrophoresis patterns revealed a band at approximately 30-50 KDa and an increase in another band at 60 KDa, probably due to the *de novo* synthesis of some gland proteins. The submandibular gland synthesizes several protein types, among which are the proline-rich proteins (PRPs) of approximately 40 KDa in molecular weight. PRPs have been associated to cell proliferation, characteristic of carcinogenesis (33). However, molecular studies are required to determine the protein type that increases during this process of tumor development.

At present, a large number of studies are devoted to the detection of oncoproteins associated to tumors that are highly prevalent in the world population (35). However, the potential association between these proteins and early malignant changes in salivary gland tumors has not been unequivocally established. The present data showed early (7 days) cytoplasmic immunostaining for p53 in response to the carcinogen DMBA in duct cells of normal areas of the glands. Immunohistochemical studies of p53 are based on the detection of the wild and mutant forms of the gene. The mutant form has a longer half-life than the wild-type. The accumulation of the mutant form of p53 is generally detected in the cell nucleus. Further studies such as those performed in neuroblastoma, mammary and colon tumors (5,11), provide evidence for the cytoplasmic expression of this protein (11) in keeping with the present findings. At 30 and 150 days, immunolabeling was positive in the nucleus. No statistically significant differences were found in staining between both time-points. This finding would indicate the appearance of molecular changes at early stages of the carcinogenesis process in the gland. Conversely, no DNA mutations were observed in the amplified region of p53 at any of the time-points evaluated. Different head and neck sites exhibit different p53 mutations and in 40% of the cases there is no correlation between immunocytochemistry and gene sequence (35,36). The nuclear detection of p53 is due to the accumulation of a mutant protein. However, occasionally, tumors that are histochemically positive for p53 do not exhibit mutations. These false positive cases could be due to the stabilization of p53 by binding to other proteins such as Mdm2 or to the detection of physiological amounts of normal p53 protein derived from an overregulation in response to DNA damage (34). This suggests that post-translation alterations evidenced by immunocytochemistry might be significant in some tumors and could be independent of the presence of genetic alterations.

The positive cytoplasmic immunolabeling of Bcl-2, an anti-apoptotic protein, at all the time-points evaluated, would indicate a reduction in the apoptotic mechanisms and an ensuing abnormal cell survival. The rise in Bcl-2 expression was greater for the longer times (Table 2). The rise in cytoplasmic levels of Bcl-2 expression has been demonstrated in several human tumors such as neuroblastomas, linfomas, melanomas, breast cancer, meningiomas and gastrointestinal carcinomas (12,13). In some of these tumors, overexpression of Bcl-2 was associated to bad prognosis coupled to other parameters such as localization, differentiation of the primary tumor and histological type (38). However, to date, the evidence for the use of Bcl-2 as a marker of malignant change is controversial (6). In keeping with morpho-physiological and immunocytochemical evidence, at all the time points evaluated, we detected a mutation in the amplified region of Bcl-2 in 50% of the glands that corresponded to an aminoacid change (Thre→Asn) in the protein. Conceivably, this might affect the insertion of the protein in the mitochondrial membrane and thus alter its function. This phenomenon

was observed for the mutation of threonine 169 of Bax in the membrane anchor domain, affecting the normal function of the protein (37).

The choice of regions of the genes evaluated was based on the scarce literature available on mutations in head and neck cancer (39). According to the latest reports of the WHO (2005) to date there is no unequivocal data on which are the oncogenes that most influence salivary gland tumorigenesis. The gene TP53 has been identified as one of the mutated genes in this pathology but its molecular epidemiology has not yet been fully established whereas in breast, colon and bladder tumors molecular epidemiology data are available (40). Within this context, the early positive immunolabeling reported herein for proteins p53 and Bcl-2 during the first stages of histopathological modifications are particularly contributory.

The histopathological, biochemical, immunocytochemical and genetic modifications observed in experimental carcinogenesis of the rat submandibular gland could represent reproducible indicators of malignant transformation that can be extrapolated to human pathology, given the high degree of homology of these oncogenes between rat, mouse and man.

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