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# Sequential Expression of Inducible Nitric Oxide Synthase and Cyclooxygenase-2 during DMBA-induced Hamster Buccal Pouch Carcinogenesis

SOO-A KIM[1], SANG-GUN AHN[1], DO KYUNG KIM[1], SU GWAN KIM[1], SANG HO LEE[1], JIN KIM[2] and JUNG HOON YOON[1]

[1]*Oral Biology Research Institute, BK 21 Project, Chosun University School of Dentistry, Gwangju;*
[2]*Department of Oral Pathology, Oral Cancer Research Institute, Yonsei University College of Dentistry, Seoul, Korea*

**Abstract.** *Background: Although it is known that iNOS and COX-2 are abundantly expressed in oral premalignant and malignant lesions, respectively, the interaction between iNOS and COX-2 has not been extensively studied. The purpose of this study was to examine the alteration of the iNOS and COX-2 expression level during hamster buccal pouch (HBP) carcinogenesis. Materials and Methods: The expression of both iNOS and COX-2 on normal, dysplastic mucosa and squamous cell carcinoma (SCC) from different differentiation stages in 7, 12-dimethylbenz[a]anthracene (DMBA)-induced HBP carcinogenesis was examined using immunohistochemical analysis. Results: The mean values of both iNOS and COX-2 expression increased gradually from control to dysplastic lesions and more to invasive SCC. The highest mean expression was SCC. The differences between both iNOS and COX-2 expression in the normal and that in the dysplastic and carcinoma lesions were statistically significant. Conclusion: The results suggest that iNOS can enhance its ability to promote tumor growth in cooperation with COX-2. The expression of iNOS and COX-2 may be one of the factors that contribute to oral carcinogenesis.*

Nitric oxide (NO) is a short-lived, soluble, free radical gas produced by a variety of cells and capable of mediating a bewildering numbers of effector functions (1). It is derived from the amino acid L-arginine in a reaction catalyzed by three different isoforms of nitric oxide synthase (NOS): endothelial nitric oxide synthase (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (2). iNOS produces larger quantities of NO, which is involved in inflammation and tumorigenesis. Besides acting as an initiator of carcinogenesis, NO is involved in the promotional stage of tumorigenesis or neoplastic transformation (3).

Like iNOS, COX-2 is an inducible enzyme that is not found in normal conditions, but is induced by a variety of pathophysiological conditions of tissues by growth factors, inflammatory stimuli, oncogenes and tumor promoters (4,5). Several studies show increased levels of COX-2 in premalignant and malignant lesions, while genetic evidence also implicates COX-2 in tumorigenesis (6-8).

There has been an increasing body of evidence supporting the roles of endogenous or exogenous NO in prostaglandin (PG) biosynthesis. Under certain pathophysiological conditions, NO and prostanoids appear to work cooperatively and synergistically (9,10). Recent studies indicate the NO has a profound effect on COX-2 catalytic activity and NO increases COX-2 activity (9,10).

Oral squamous cell carcinoma (OSCC) develops as a multistep process by the accumulation of genetic and epigenetic changes in the field exposed to the carcinogen (11,12). This hypothesis is supported by the frequent presence of precancerous lesions in the epithelial field of oral cancers and the high frequency of second primary tumors in patients definitively treated for their first oral primary tumor (13). Histologically, carcinogenesis of the oral mucosa is progression from normal epithelium to hyperplastic, dysplastic lesions and invasive carcinoma (14). The hamster buccal pouch (HBP) mucosa model is one of the most widely accepted experimental models of human oral cancer (15-17). Although anatomical and histological differences between HBP and human oral mucosa are observed, carcinogenesis protocols induce premalignant and malignant changes that recapitulate many of the features observed during human oral carcinogenesis (15-17). In addition, this model is used to study the biochemical and molecular events leading to oral cancer (17).

*Correspondence to:* Jung Hoon Yoon, DDS, PhD, Department of Oral Pathology, Chosun University School of Dentistry, #375 Seosuk-dong, Dong-gu, Gwangju 501-759, Korea. Tel: +82-62-230-6879, Fax: +82-62-223-3205, e-mail: jhyoon@chosun.ac.kr

*Key Words:* iNOS, COX-2, oral cancer, DMBA, hamster buccal pouch carcinoma model, carcinogenesis.

Table I. *The mean values of iNOS and COX-2 expression during DMBA-induced hamster buccal pouch carcinogenesis.*

| | Control | 2W | 4W | 6W | 8W | 10W | 12W | 14W |
|---|---|---|---|---|---|---|---|---|
| Histology | NM | Hyp | MiD | MD | SD | CIS | SCC | SCC |
| iNOS | 0.01±0.005 | 1.8±0.92 | 4.4±0.51 | 4.2±0.58 | 5±0.95 | 5.6±0.4 | 5.8±0.37 | 6±0.32 |
| COX-2 | 0.0005±0.007 | 0.8±0.49 | 4.8±0.58 | 4.8±0.58 | 6±0.32 | 5.6±0.4 | 6±0.32 | 6.2±0.37 |

NM: Normal mucosa, Hyp: Hyperplasia, Mid: Mild dysplasia, MD: Moderate dysplasia
SD: Severe dysplasia, CIS: Carcinama *in situ*, SCC: Squamous cell carcinoma, Mean ± S.E.M.

Both iNOS and COX-2 are abundantly expressed in oral premalignant lesion and associated OSCC, respectively (18-21). However, the interaction between iNOS and COX-2 has not been extensively studied. In particular, the expression of COX-2 has not been previously studied in chemically-induced oral carcinogenesis. In this study, therefore, we investigated the interactions between iNOS and COX-2 during 7,12-dimethylbenz[a]anthracene (DMBA)-induced hamster buccal pouch carcinogenesis.

## Materials and Methods

*Animals and treatments.* Six-week-old male Syrian golden hamsters, which weighed approximately 120 g at the beginning of the experiment, were obtained from KIST (Taejon, Korea). The animals were housed under controlled conditions (22˚C, 12-h light/dark cycle) and given standard laboratory chow and tap water *ad libitum*. After allowing the animals to acclimatize for one week, both pouches of 42 hamsters were painted with a 0.5% DMBA (D-3254; Sigma Chemical Co., St. Louis, MO, USA) solution dissolved in mineral oil (USP), three times weekly for 14 weeks, using a cotton pestle. The bilateral pouches of 2 hamsters of the control group were similarly treated with 0.2 ml of mineral oil alone. The last group of 7 hamsters was untreated throughout the experiment. All hamsters were sacrificed under ether anesthesia 15 weeks after commencing the experiments. Both pouches were excised and the specimen was fixed in 10% neutral buffered formalin and processed for the histological and immunohistochemical examinations.

*Immunohistochemistry.* Immunostainings were performed by a standard avidin-biotin peroxidase complex (ABC) method. The previously fixed tissues were embedded in paraffin. Five-micrometer specimens were made, which were mounted on poly-L-lysine-coated glass slides and dried overnight at room temperature. After the sections had been deparaffinized in xylene and rehydrated using graded ethanol, they were immersed in 3% hydrogen peroxide in methanol (V/V) for 15 min in order to quench the endogenous peroxidase activity. They were then washed in TRIS buffer and incubated with normal 1% BSA (bovine serum albumin in TRIS buffer) for 1 h to reduce the nonspecific binding of the primary antibody. After washing in TRIS buffer, the tissues were stained for iNOS protein using a primary rabbit polyclonal antibody (Calbiochem, Cat. No. 482728) and COX-2 protein using a primary monoclonal anti-PGHS-2 (Transduction Laboratories, Lexington, KY, USA) at 1:200 dilution overnight in a humidified chamber at 4˚C. They were then incubated for 30 min at room temperature with biotin-conjugated goat anti-rabbit IgG (Vector, Burlingame, USA; 1:100) and then for 30 min with ABC (DAKO, Santabara, USA). To detect the immunoreactivity, the sections were treated with diaminobenzidine (0.8 mM) and counterstained with hematoxylin. Negative controls for the specificity of anti-iNOS and anti-PGHS-2 antisera were included by omitting the primary antisera.

*Evaluation.* The intensity of both iNOS and COX-2 in the epithelium and tumor cells was evaluated by classifying into four groups: 0 = no; 1 = mild; 2 = moderate; and 3 = strong staining intensity. The area of immunostainings was evaluated as follows: 0 = no positive immunostaining, 1 = <10%; 2 = 10-25; 3 = 25-50; and 4 = >50% showing positive immunoreactivity. A combined score for both iNOS and COX-2 immunostainings, based on both qualitative and quantitative immunostaining, were composed by adding the qualitative to the quantitative score. The maximum score after adding was 7 and the minimum, 0. The results are presented as mean±S.E.M. Statistical significance was analyzed by using the Student's *t*-test for two groups and one way analysis of variance for multi-group comparison. $P<0.05$ is considered statistically significant.

## Results

*Histopathology.* As shown in Table I, the gross and histopathological changes in the DMBA-treated HBP mucosa were similar to those described in our previous study (16). Areas of dysplasia and invasive SCC with a 100% tumor incidence developed in all of the DMBA-treated HBP mucosa. The untreated- and mineral oil-treated pouches showed no obvious changes, whereas the mild hyperkeratosis and acanthosis were found in the 2-week-DMBA-treated pouches. Mild dysplastic changes were first observed at week 4. The severity of the dysplasia increased in the following experimental periods. The first histological evidence of SCC was noted in week 10 and invasive SCC was noted in week 12 (Table I).

*Immunohistochemical analysis.* No iNOS activity could be detected in the untreated or mineral oil-treated pouches. Both cytoplasmic and nuclear stainings were observed in the DMBA-treated HBP mucosa. There was also iNOS

expression in the stromal cells, including endothelial and inflammatory cells. The mean values of iNOS expression increased gradually from control to dysplastic lesions and more to invasive SCC (Figure 1A,C,E). The highest mean expression was SCC at week 14 (Table I). The differences between iNOS expression in the normal and that in the dysplastic and carcinoma lesions were statistically significant (Figure 2).

Normal lesions do not express substantial quantities of COX-2. COX-2 protein is cytoplasmically detectable in lesional epithelial cells, endothelial cells and inflammatory cells. The immunoreactivity for COX-2 protein increased as the tissue passed from hyperplasia to dysplasia and SCC (Figure 1B,D,F). The highest mean expression was SCC at week 14 (Table I). The differences between COX-2 expression in the normal and that in the dysplastic and carcinomatous lesions was statistically significant (Figure 3).

## Discussion

In the current study, both iNOS and COX-2 immunoreactivities were proportional to the progression of carcinogenesis, from epithelial hyperplasia through dysplasia and finally to invasive carcinoma. These results suggest that high iNOS and COX-2 activity may be closely linked to chemically-induced multistage oral carcinogenesis. In addition, both iNOS and COX-2 proteins have an important role in both the early and the later stages of oral epithelial carcinogenesis. It has been shown that both iNOS and COX-2 proteins were expressed in premalignant and malignant, but not normal oral and laryngeal mucosa, suggesting that it is an immunohistochemical marker for upper aerodigestive tract SCC (18,19,22-24). Our data is in agreement with the results of the previous studies, documenting a progressive increase in both iNOS and COX-2 expression in the transition from normal mucosa through dysplasia to invasive carcinoma, both in hamster and human oral cancers (18,19,22,25-28).

Excess NO could play a role in carcinogenesis by impairing the tumor-suppressor function of *p53* (29). In addition, wild-type but not mutant *p53* suppressed *COX-2* transcription, raising the possibility that *p53* status is also a determinant of COX-2 expression (30). Overexpression of COX-2 alters cell adhesion and inhibits apoptosis by increasing the Bcl-2 protein production (31). For *p53*-mutant cells, the overexpression of iNOS and COX-2 would be unchecked and hence an up-regulation of NO and PGs would subsequently occur, with the potential of leading to a cancerous state (29-32). In the DMBA-induced HBP model, p53 protein accumulation occurs frequently and early in carcinogenesis, as it does in human SCCs, and some of these p53 alterations are due to *p53* gene mutations (15). Therefore, it is believed that the loss of wild-type p53 function or the expression of mutant *p53* in the tumor would permit both the apoptosis and growth of the tumor in the presence of NO and PGs. In this study, both the iNOS and COX-2 expression level was the highest at the last stage, SCC at week 14. This is consistent with the hypothesis that a progressive increase of iNOS and COX-2 levels could contribute to DMBA-induced HBP carcinogenesis by inducing an accumulation of abnormal epithelial cells in the invasive carcinomas. However, it was not elucidated whether *p53* mutation might result from either NO and PGs itself or DMBA-induced genotoxicity. Recent observations support a correlation between p53 and iNOS or COX-2 expression for human oral epithelial dysplasia and OSCC (18-21). Cells that carry mutant p53, especially in dysplastic lesions and OSCCs, express high levels of iNOS and COX-2 (20,21).

Co-induction or co-regulation of iNOS and COX-2 enzymes has been observed under diverse experimental conditions (3). Since both genes are co-regulated by the inflammatory mediator transcription factor NF-kappa B, the same pathophysiological stimuli may turn on the expression of both genes simultaneously (3,33). Alternatively, NO produced through NF-kappa B-induced iNOS expression or *via* another pathway independently of NF-kappa B, may affect COX-2 expression/catalytic activity (3). In the current study, we demonstrated that the expression level of iNOS together with COX-2 was up-regulated in the dysplastic lesion and SCC compared to normal and hyperplastic mucosa. Thus, there is the possibility that the ability of iNOS to promote tumor growth is enhanced in cooperation with COX-2. However, we could not find the mechanisms of interaction between iNOS and COX-2.

Overall, the present study demonstrated the sequential expression of iNOS and COX-2 in DMBA-induced HBP carcinogenesis. Whatever the mechanisms of interaction, this finding indicates a possible association of iNOS with COX-2 in the development of chemically-induced oral carcinomas. The expression of iNOS and COX-2 may be one of the factors that contribute to oral carcinogenesis. However, further study regarding the mechanisms of the apparent activation of COX-2 by NO is deemed necessary in order to evaluate the exact role of iNOS and COX-2 in this DMBA-induced HBP carcinogenesis model.

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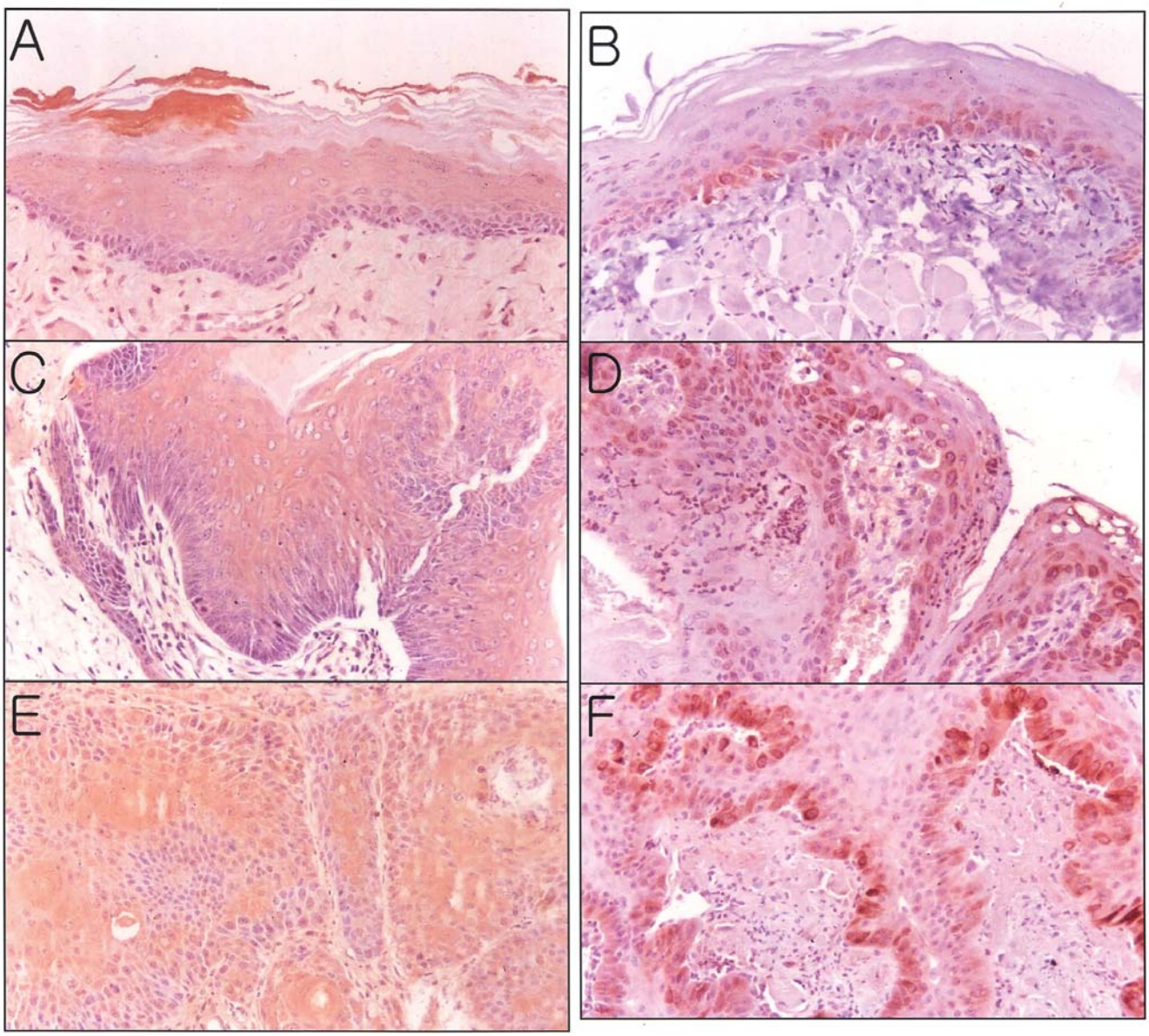


Figure 1. *Immunohistochemical iNOS and COX-2 stainings during DMBA-induced hamster buccal pouch carcinogenesis. Four-week-DMBA-treated mild epithelial dysplasia (A, B), eight-week-DMBA-treated severe epithelial dysplasia (C, D), twelve-week-DMBA-treated invasive SCC (E, F) show increasing iNOS (A,C,E) and COX-2 (B,D,F) immunoreactivity in the entire tumor via hamster buccal pouch malignant progression.*

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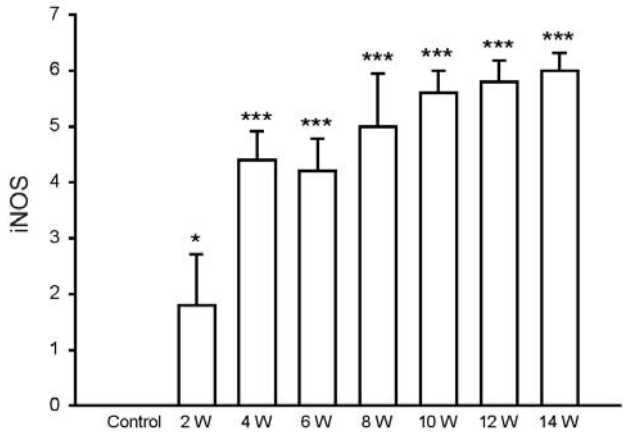


Figure 2. *The mean values of iNOS expression. The iNOS expression increased gradually from control to dysplastic lesions and more to invasive squamous cell carcinoma. The difference between iNOS expression in the normal and that in the dysplastic and carcinomatous lesions is statistically significant. * $p<0.05$ and *** $p<0.001$ vs. control (the mineral oil-treated and untreated control tissues).*

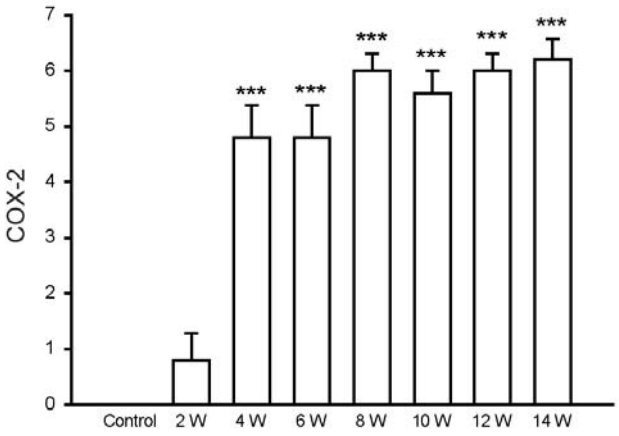


Figure 3. *The mean values of COX-2 expression. The COX-2 expression increased gradually from control to dysplastic lesions and more to invasive squamous cell carcinoma. The difference between COX-2 expression in the normal and that in the dysplastic and carcinomatous lesions is statistically significant. *** $p<0.001$ vs. control (the mineral oil-treated and untreated control tissues).*


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