Induction of apoptosis and cell cycle arrest by NS398 in oral squamous cell carcinoma cells via downregulation of E2 promoter-binding factor-1

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Abstract. Overexpression of cyclooxygenase-2 (COX-2) plays an important role in development and progression of different human cancers, but the underlying molecular mechanisms remain to be defined. Tissue specimens of normal oral epithelia (n=9), dysplasia (n=38), and oral squamous cell carcinoma (SCC, n=54) were immunohistochemically analyzed for COX-2 and E2F-1 expression. A human oral SCC cell line, Tca8113, was used to assess NS398 antitumor activity. PGE_2 levels were measured by using radioimmunoassay, and COX-2, pRb, and E2F-1 proteins were determined by Western blot assay. We found expression of COX-2 and E2F-1 proteins was significantly increased in both oral dysplasia and SCC compared to the normal epithelium. Increased COX-2 expression was associated with E2F-1 expression in both oral dysplasia and SCC. NS398 treatment reduced viability of Tca8113 cells in a dose- and time-dependent manner. NS398 suppressed PGE_2 levels, a product of COX-2 enzyme, in the tumor cells. The reduced cell viability is due to induction of apoptosis by NS398, which activates caspase-3, but does not inhibit bcl-2. NS398 also induced tumor cell arrest at G1 phase of the cell cycle and inhibited expression of COX-2, pRb and E2F-1 proteins. This study provides evidence that E2F-1 and COX-2 are overexpressed in oral cancer, and further supports suppression of COX-2 in control of oral cancer.

Introduction

Oral squamous cell carcinoma (SCC) is one of the most common malignancies in the world, with high incidence and mortality rate in the Asian oral cancer belt (1,2). Tobacco smoke and alcohol consumption are significant risk factors for oral squamous cell carcinoma, and chronic irritants such as mouthwash and poor dental hygiene have also been considered as etiological factors. Despite advancement in various treatment modalities, including surgical intervention, radiotherapy and chemotherapy, the survival rate of patients with oral SCC has not been significantly improved (1,2). Therefore, more effective approaches for prevention and treatment are urgently needed.

Cyclooxygenase-2 (COX-2), a rate-limiting enzyme in the prostaglandin synthesis pathway, is one of the important cellular factors in promoting cancer development and progression. Indeed, epidemiological and experimental studies have demonstrated increased expression of COX-2 enzyme in cancers of the colon, stomach, breast, esophagus, lung, pancreas and brain (3-9). The effectiveness of non-steroidal anti-inflammatory drugs (NSAIDs) in prevention of human cancers, including oral SCC, has been proven *in vitro* and *in vivo* (10-13). However, the underlying molecular mechanisms by which NSAIDs prevent human cancer are not well defined.

Many studies have shown that NSAIDs have the ability to alter cell cycle kinetics in a number of human tumor cells, and cell cycle alteration seems to be a major contributing factor in NSAID-mediated cancer toxicity (14,15). This implies that the actual mechanism underlying the antiproliferative activity of NSAIDs may be related to some cellular factors involved in cell cycle inhibition. E2 promoter-binding factor-1 (E2F-1), was the first cloned and is the best characterized member of this gene family (16,17). E2F-1 plays a pivotal role in regulating the expression of genes involved in the G1-S transition and DNA synthesis (18), and is the most well-known transcription factor regulated by the cyclin/Cdk/Rb pathway. Several lines of evidence indicate that E2F-1 is involved in neoplastic development. Recent studies showed that deregulation of the Rb/E2F complex results in increased COX-2 expression and activity in prostate epithelial cells (19), and that celecoxib, a COX-2-selective inhibitor, profoundly inhibits nuclear E2F function in head and neck squamous cell carcinoma, leading to G1 phase accumulation (20). These findings indicated a correlation

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between NSAID-induced cell cycle inhibition and the Rb/E2F pathway. However, very few studies have confirmed these mechanistic patterns in various tumor cells. In this study, we detected COX-2 and E2F-1 expression in tissue specimens of normal oral epithelia, dysplasia, and SCC in a Chinese patient population. We then assessed anti-proliferation activity and cell cycle inhibition by NS398, a COX-2-selective inhibitor, in a human oral SCC cell line Tca8113 and explored the underlying molecular mechanisms.

Materials and methods

Tissue specimens. Tissue samples used in this study consisted of 38 cases of oral dysplasia and 54 cases of oral SCC, which were collected between January 2002 and December 2004 in the Division of Oral and Maxillofacial Surgery, Stomatological Hospital, Nanjing Medical University. An additional nine cases of normal oral mucosae were obtained from healthy volunteers in the Department of Dental Implant Surgery. All patients signed an informed consent to use their tissue specimens for research.

All specimens were fixed in 10% buffered formalin and embedded in paraffin. Sections (3 μm) were then prepared and pathological diagnoses were made on the basis of hematoxylin and eosin stained sections, according to the WHO classification of oral SCC. Also based on WHO criteria, oral dysplasia was graded into one of three categories: mild, moderate or severe.

Immunohistochemistry. Immunohistochemical staining was performed by using the avidin-biotin-peroxidase technique (ABC). A polyclonal anti-COX-2 antibody (Cat# sc-1746) and a monoclonal anti-E2F-1 antibody (Cat# sc-251) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Briefly, after de-paraffinization and re-hydration, the sections were treated with 3% hydrogen peroxide for 15 min to inhibit endogenous peroxidase activity. Antigen retrieval was achieved by using a microwave of the samples in citrate buffer (pH 6.0) (750 W for 5 min followed by 150 W for 15 min). The sections were then incubated with anti-COX-2 or anti-E2F-1 antibody at a dilution of 1:100 for 1 h at room temperature and the second antibody was incubated for 1 h, followed by ABC incubation for 30 min. Immunoreactions were visualized with 3,3-diaminobenzine (DAB) and the sections were counterstained with hematoxylin for 2 min. Some sections were incubated with phosphate buffered saline (PBS) solution instead of the primary antibody as negative controls.

To review and score the stained sections, we selected >1,000 cells per section for counting positively stained cells at x40 magnification in five or more microscopic fields. In sections of normal mucosa and oral dysplasia lesions, our counting began at the basal levels of the epithelium and progressed superficially. In oral SCC sections, counting was performed from peripheral to more central regions of the cancer nests.

Positively staining cells were summarized as the labeling index (LI), given as a percentage (%) of the total cells counted. The data were evaluated semiquantitatively and graded according to the following criteria: low expression, for <20% cells stained; and high expression, for >20% (21,22). All sections were assessed by two independent researchers experienced in pathologic analysis and immunohistochemistry. If a specimen was found to have differing labeling indices between the two independent readings, a final determination was made by consensus review.

Cell culture. Tca8113, obtained from Institution of Biochemistry and Cell Biology (Shanghai, P.R. China), was cultured in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 unit/ml of penicillin and 100 μg/ml of streptomycin under 5% CO_2 at 37˚C.

Cell proliferation assay. A COX-2-selective inhibitor, NS398, was obtained from Cayman Chemical Company (Ann Arbor, MI, USA) and dissolved in dimethylsulfoxide (DMSO) and stored at -20˚C as aliquots until use. To assess the effect of NS398 on Tca8113 cells, the cells ($2x10^4$ cells/well) were first seeded in 96-well, flat-bottomed plates in triplicate. Different concentrations of NS398 were added in a final volume of 200 μl of growth medium/well in the next day. DMSO was added to control wells at equal volumes of the drug. The plates were then incubated at 37˚C in a 5% CO_2-supplemented atmosphere for 24, 48, 72 and 96 h. At the end of the experiments, the media were removed and replaced by 150 μl of 0.5 μg/ml of MTT (Sigma-Aldrich Chemical Co., USA) in the growth medium, and the plates were then incubated for 4 h at 37˚C. The reduced MTT dye was solubilized with 150 μl DMSO per well. The optical density (OD) values were read with an ELX800 Universal Microplate reader (Bio-Tek Co., CA, USA) at 570 nm. Percentage of inhibition was calculated as: % inhibition = 1 - (ODtest/ODcontrol) x 100%.

Flow cytometric analysis. After treatment with NS398 for different periods of time, cell suspensions were prepared by trypsinization and $1x10^6$ cells/ml were washed with PBS and then fixed overnight in 10 ml of 70% ethanol (-20˚C) at 4˚C. After that, the cells were incubated with RNase at a concentration of 0.25 μg/ml at 37˚C for 1 h, then incubated with propidium iodide (50 μg/ml in PBS) for 30 min at 4˚C in the dark. Before flow cytometry analysis, the samples were passed through a 25-gauge needle to prevent nuclear clumps. The samples were incubated in the dark at 37˚C for 30 min. The cells were then analyzed with a FACSCalibur flow cytometer (Becton-Dickinson, CA, USA). For each sample, 10,000 fluorescent cells were counted. The data were analyzed using ModFit software.

Western blot analysis. After treatment with NS398, the conditioned media were removed and stored at -70˚C to assess PGE_2 levels. Protein lysates from Tca8113 cells were obtained by washing the cells 3 times with ice-cold PBS and scraped in ice-cold lysis buffer (1% NP-40, 1% NaTDC, 0.1% SDS, 0.15 M NaCl, 0.01 M Na_3PO_4, 2 mM EDTA, 50 mM NaF). Protein concentrations were determined by using GeneQuant Pro protein assay (GE Healthcare, Piscataway, NJ, USA). Cell extracts (100 μg) were separated by electrophoresis on 12% SDS polyacrylamide gel (PAGE). The protein was then transferred by electroblotting onto a polyvi-

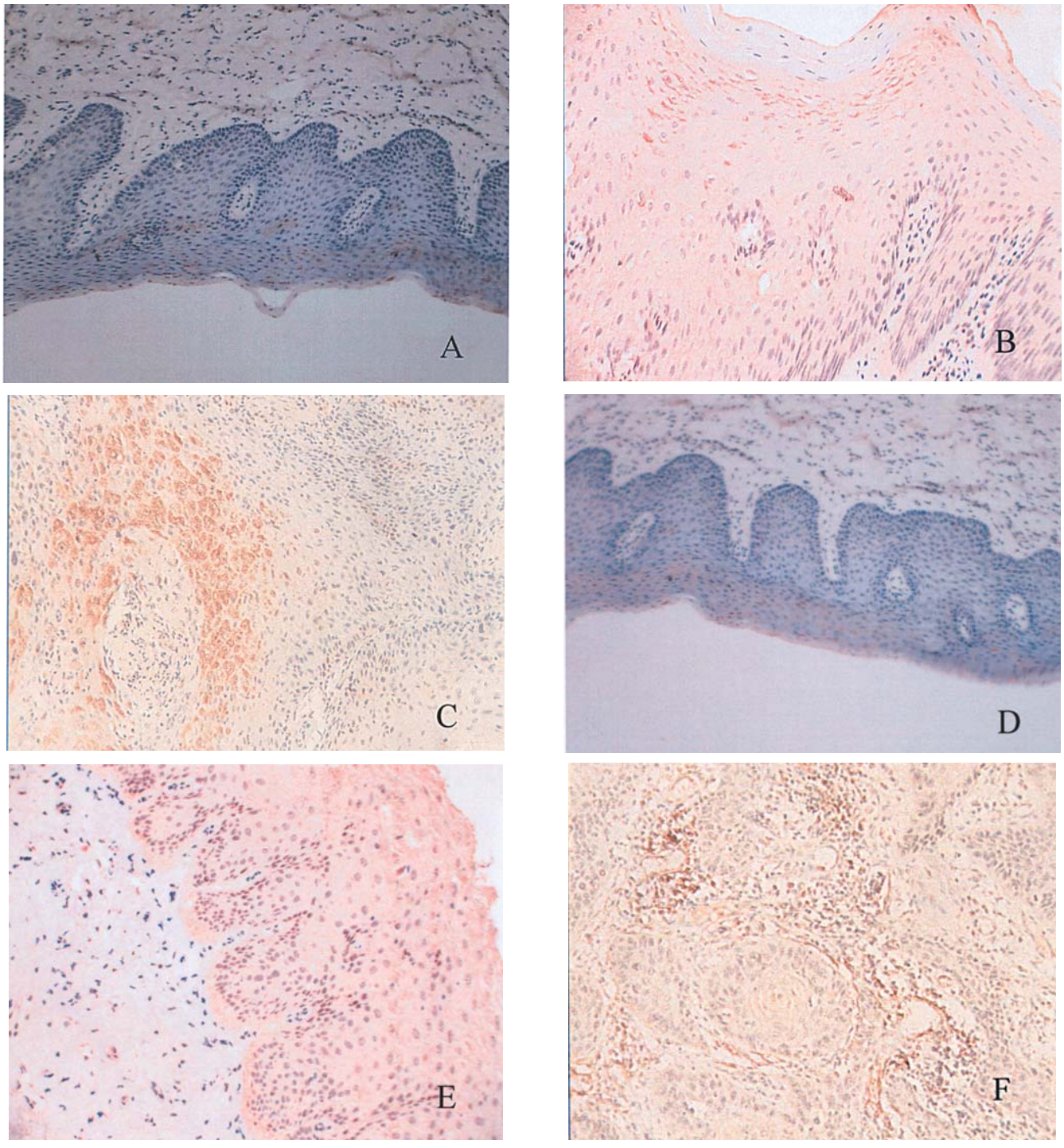

Figure 1. Immunohistochemical detection of COX-2 and E2F-1 proteins in normal oral epithelium, dysplasia and SCC (original magnification, x200). (A) COX-2 expression in normal epithelium; (B) COX-2 expression in oral dysplasia; (C) COX-2 expression in oral SCC; (D) E2F-1 expression in normal epithelium; (E) E2F-1 expression in oral dysplasia; and (F) E2F-1 expression in oral SCC.

nylidene difluoride membrane. After 1-h incubation in blocking solution (5% non-fat dry milk and 0.1% Tween-20 in PBS), the membrane was blotted with an antibody against COX-2, E2F-1, pRb, bcl-2, or caspase-3 (all were from Santa Cruz Biotechnology), or a monoclonal anti-ß-actin antibody (Boster, Wuhan, P.R. China) overnight at 4˚C. After being washed with PBS-T, the blots were incubated with peroxidase-labeled secondary antibodies (Boster) for 1 h at 37˚C. The specific bands were detected by using an enhanced chemiluminescence system (Pierce, IL, USA). The intensity of protein bands was detected by densitometric scanning and reported in band intensity units. The relative abundance of each target was normalized by ß-actin.

PGE_2 measurement. PGE_2 levels in the cells before and after treatment with NS398 were assayed in the conditioned media previously frozen at -70˚C by using a PGE_2 (I^{125}) RIA kit (Suzhou University, Suzhou, P.R. China) according to the manufacturer's instructions. The levels of PGE_2 were then normalized by protein concentration.

Table I. Labeling indices of E2F-1 and COX-2 in oral normal epithelium, dysplasia and oral squamous cell carcinoma.

	Cases	COX-2	E2F-1
Normal	9	2.22±0.70	3.78±1.53
Dysplasia	38	38.05±2.59[a]	38.03±2.20[a]
Mild	11	30.00±3.86[a]	27.55±1.88[a]
Moderate	14	35.21±3.72[a]	36.21±2.75[a]
Severe	13	43.92±4.51[a]	48.85±3.81[a]
SCC	54	44.17±3.62[a]	36.09±2.26[a]
Well	24	48.13±5.82[a]	43.04±3.57[a]
Moderate	22	44.82±5.87[a]	33.23±3.05[a,b]
Poorly	8	35.50±4.43[a]	23.13±3.25[a,b]

Mean ± SD. [a]p<0.01, compared to normal epithelium; [b]p<0.05 compared to dysplastic lesions.

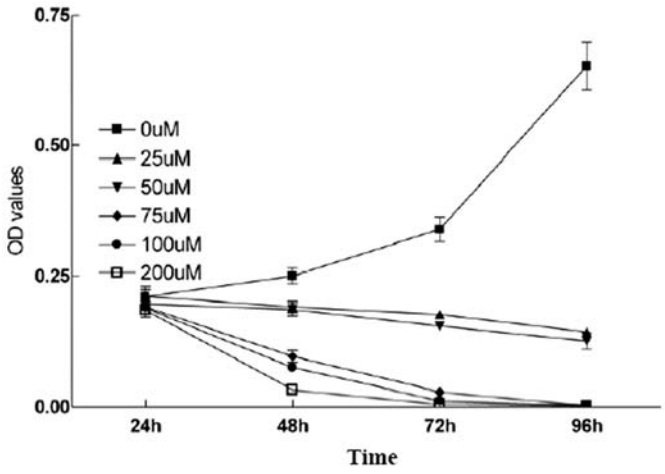

Figure 2. Inhibitory effect of NS398 on proliferation of oral SCC cells. Tca8113 cells were cultured in RPMI-1640 medium containing NS398 at concentrations of 0, 25, 50, 75, 100, or 200 μM for up to 96 h. Cell viability was measured by MTT assay and the optical density (OD) values were determined. The experiments were in triplicates and repeated three times with similar results.

Statistical analysis. The experimental data were presented as means ± SD. Statistical analyses included use of a 2-tailed Student's t-test or one-way analysis of variance (ANOVA). The correlation between E2F-1 and COX-2 was assessed by using Spearman's correlation coefficient test. Statistical significance was set at $p \leq 0.05$.

Results

Expression of COX-2 and E2F-1 in normal oral mucosae, dysplasia and oral SCC. Positive immunostaining of COX-2 was seen in the cytoplasm of the epithelial cells and the staining pattern was dot-like. COX-2 was weakly expressed in the basal layer of the normal epithelium, but significantly increased in the dysplastic and carcinoma cells (Fig. 1A-C). In particular, 28 of 38 samples (73.7%) of dysplastic lesions and 44 of 54 (81.5%) of oral SCC showed positive cytoplasmic staining of COX-2 protein. In oral SCC, the peripheral portion of tumor nests expressed more COX-2 protein than the center of tumor nests. Overall, the mean labeling indices (LI) of COX-2 were 2.22±0.70 in normal oral epithelia, 38.05±2.59 in the dysplastic lesions, and 44.17±3.62 in oral SCC (Table I). The difference was statistically significant between the normal and oral SCC (p<0.01) and between the normal and dysplastic tissues (p<0.01). The severe dysplasia lesions and the well differentiated tumors tended to have higher COX-2 expression; however, there was no correlation found between the LIs and the histological grades.

In contrast, the immunoreactivity of E2F-1 was found in the nuclei of the epithelia, although in some cases there was some staining in the cytoplasm (Fig. 1D-F). The mean LIs of E2F-1 for normal epithelia, dysplasia and SCC were 3.78±1.53, 38.03±2.20 and 36.09±2.26, respectively. The LIs in moderate and severe dysplastic lesions were significantly higher than mild lesions in oral dysplasia (p<0.05), while in oral SCC the LIs of well differentiated tumors were significantly higher than the moderate and poorly differentiated cancers (p<0.05).

Furthermore, 28 samples (73.7%) of dysplasia and 41 samples (75.9%) of oral SCC expressed high levels of both E2F-1 and COX-2 proteins (p<0.01, by Spearman's coefficient test, r=0.67).

NS398 inhibition of tumor cell proliferation. MTT assay showed that NS398 significantly reduced viability of Tca8113 cells in a dose- and time-dependent manner (Fig. 2). After treatment with 50 μM of NS398, the cell viability was reduced to 29.7, 54.7 and 81.51% after 48, 72 and 96-h culture, respectively. At 100 μM of NS398, the tumor cells were almost unviable.

NS398 induction of apoptosis in Tca8113 cells. To determine whether the reduced cell viability by NS398 in Tca8113 cell line was due to induction of apoptosis, we performed TUNEL assay. Fig. 3 shows that NS398 did induce these cells to undergo apoptosis. Western blot analysis showed that caspase-3 expression was induced, but bcl-2 expression had no change (Fig. 4). Furthermore, data from DNA histograms of a flow cytometric analysis showed a reduced number of cells in the S and G2-M phases, but accumulation in G1 phase after the tumor cells were treated with 50 μM of NS398 for different periods of time (Fig. 3).

NS398 inhibition of PGE_2 production. After treatment with NS398, PGE_2 levels were significantly reduced compared to the controls for the duration of treatment, demonstrating an inverse relationship between PGE_2 production and duration of treatment, i.e., PGE_2 concentrations (pg/mg protein) in the conditioned media were 26.56 at 6 h, 16.61 at 12 h, 10.42 at 24 h, 2.07 at 48 h and 0.98 at 72-h treatment (Fig. 5).

Effect of NS398 on gene expression in Tca8113 cells. We next analyzed gene expression after NS398 treatment. We

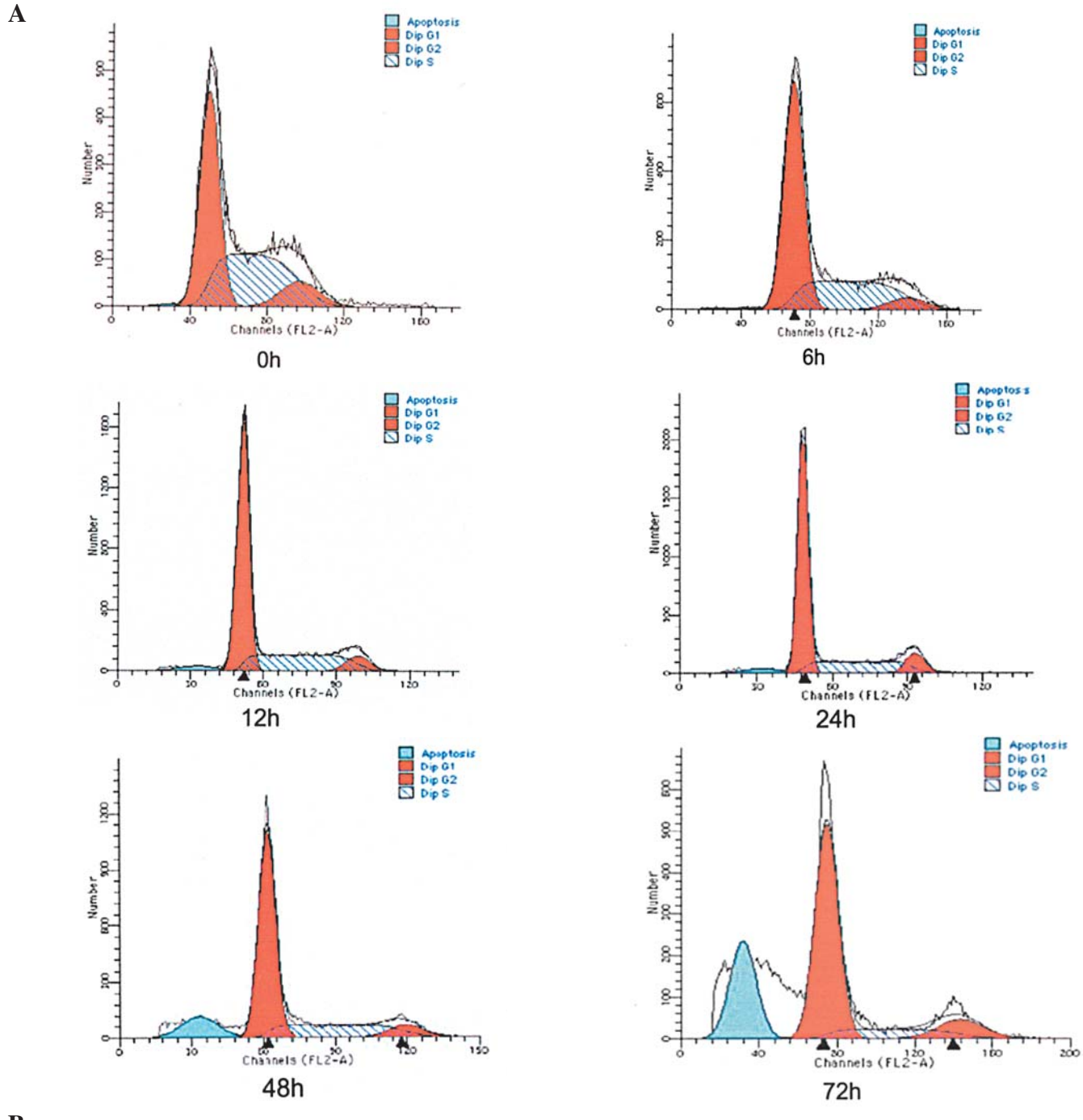

Figure 3. Effect of NS398 on cell cycle arrest and apoptosis in oral squamous cell cancer cell line after treated with 50 μM of NS398 for up to 72 h. The distribution of cell cycles (A) and apoptosis rate (B) was measured by flow cytometry (see details in Materials and methods).

found that NS398 treatment inhibited COX-2 expression in Tca8113 cells compared to the control ($p<0.01$) (Fig. 4). Meanwhile, expression of pRb and E2F-1 proteins was also suppressed by NS398 treatment in Tca8113 cells (Fig. 4).

Discussion

In the present study, we analyzed expression of COX-2 and E2F-1 proteins in tissue specimens that represent the multiple stages of oral carcinogenesis - from normal and dysplasia to SCC. We found a significant increase in COX-2 and E2F-1 expression in oral dysplasia and SCC. We then performed a series of experiments to assess whether inhibition of COX-2

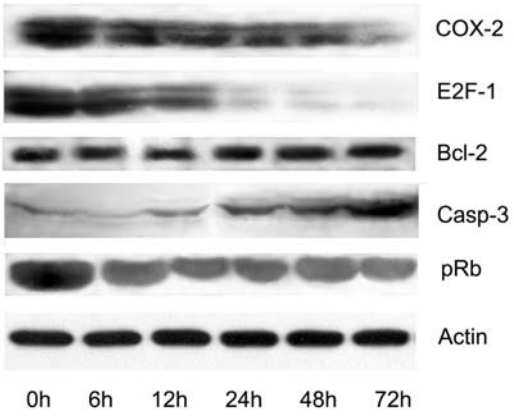

Figure 4. Western blot analysis of gene expression. Tca8113 cells were cultured in RPMI-1640 medium and treated with 50 μM of NS398 for different periods of time. The total cellular protein was isolated and subjected to Western blot analyses of different gene expression. Quantified densities of bands were expressed as ratios to the densities of the corresponding ß-actin. The data were expressed as mean ± SD. The experiment was repeated once with similar data.

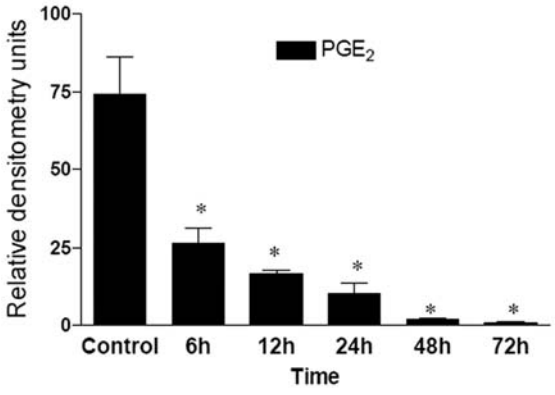

Figure. 5. Suppression of PGE_2 production by NS398 treatment in oral SCC cells. Tca8113 cells were cultured in RPMI-1640 medium and treated with 50 μM of NS398 for up to 72 h. The conditioned growth medium was used to measure PGE_2 levels by using a PGE_2 kit (see the Materials and methods). $^*p<0.01$ vs. control.

could be used to control oral cancer. We found that NS398 significantly inhibited viability of oral SCC cells and induced them to undergo apoptosis, which was accomplished through induction of caspase-3 activation and cell cycle arrest at G1 phase. NS398 also suppressed expression of E2F-1 and pRb, but not Bcl-2. The results of this study indicate that suppression of COX-2 enzymatic activity was able to inhibit E2F-1 expression and Rb phosphorylation, which may have potential in the control of oral SCC in future clinical practice.

A number of studies have shown an association between COX-2 overexpression and anti-apoptosis features in different cancer cells. For example, Tsujii and DuBois (25) first showed that transfection of the COX-2 gene into rat intestinal epithelial cells reduced the susceptibility to apoptosis and up-regulated the expression of anti-apoptotic Bcl-2 protein. Similar results were observed in human colon cancer cells (26). Terakado *et al* found that the level of COX-2 expression correlated inversely with increased radiation sensitivity and celecoxib, a selective COX-2 inhibitor, enhanced the radiation response in oral SCC cell line (27). Previous studies had shown that NS398 could suppress growth and induce apoptosis in a number of different cancer cell lines (9,28-30), although a study by Helena *et al* reported no significant apoptosis in the response of oral SCC cell lines to NS398 treatment except for G0/G1 cell cycle arrest (12). In the current study, we demonstrated that caspase-3 expression was enhanced after NS398 treatment, but no change in bcl-2 levels was found. This indicates that NS398 induction of caspase-3 activation results in induction of apoptosis, but is independent of bcl-2 reduction.

It has been reported that >90% of oral cancers had at least one abnormality that affects Rb, cyclin D1, or p16, which suggests that cell cycle regulation pathways may be useful targets for control of oral carcinogenesis (31). After activation by cyclin D1, CDK4 or CDK6 will phosphorylate the Rb protein, leading to its functional inactivation and release of transcription factors necessary for entry into S phase and cell cycle progression (32,33). In a recent study by Crawford *et al* (34), silencing of $p16^{INK4A}$ gene expression through promoter hypermethylation resulted in increased COX-2 levels and elevated PGE_2 in mammary epithelial cells. The increased COX-2 expression contributed to increased angiogenic activity and invasiveness. However, NS398 can reduce cell survival and induce apoptosis of mammary epithelial cells (34). Our current data demonstrated that 50 μM of NS398 inhibited growth of Tca8113 cells in a dose- and time-dependent manner and induces apoptosis. NS398 was able to reduce E2F-1 expression and inhibit Rb phosphorylation. Indeed, our *ex vivo* data also showed an association between COX-2 and E2F-1 expression, i.e., high levels of E2F-1 expression were associated with over-expression of COX-2 protein in oral dysplastic and tumor tissues.

Previous studies have reported that cell cycle arrest at G1 phase was induced by different NSAIDs (35,36). Our data also support the NS398-induced cell cycle arrest at G1 phase. Bock *et al* have reported that celecoxib toxicity is S phase specific and that inhibition of nuclear E2F-1 function is the possible mechanism (20). Our study showed that NS398 suppressed E2F-1 expression and Rb phosphorylation in an oral SCC cell line.

Development of oral SCC, like all other cancers, involves multiple genetic alterations such as oncogene activation and tumor-suppressor gene dysfunction (1,37). To date, a large body of knowledge has been generated regarding molecular alterations associated with oral carcinogenesis. Molecular biology studies of oral cancer have revealed frequent genetic abnormalities in oral SCC. These alterations include aberrant cell cycle control, cell signaling, cellular enzymes, growth factor receptors, and nuclear receptors (such as p53, p16, cyclin D1, EGFR, E-cadherin, COX-2 and RAR-ß) (1,37,38). The knowledge generated from these studies has begun to coalesce into an improved understanding of oral cancer for early detection, prevention and treatment. Targeting these molecules may help us to discover novel preventive or therapeutic strategies. Indeed, use of NSAIDs has been clinically shown to prevent head and neck cancer. This study further supports their use.

In summary, our data demonstrated that expression of E2F-1 and COX-2 were induced in oral SCC, and their association and downregulation by NS398 may represent a novel target for prevention and treatment of oral SCC.

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