Mol. Cells *29*, 185-194, February 28, 2010
DOI/10.1007/s10059-010-0026-y

Molecules and Cells

Anti-Cancer Effects of Celecoxib in Head and Neck Carcinoma

Young-Youn Kim, Eun-Jin Lee, Yu-Kyoung Kim[1], Soung-Min Kim, Ju-Yong Park[2], Hoon Myoung*, and Myung-Jin Kim

Although many studies highlighted cyclooxygenase2 (COX2) inhibition as a promising therapeutic strategy for cancer, more evidence is needed for clinical application. The purpose of this study was to investigate the feasibility of COX2 inhibition as a strategic treatment modality for head and neck carcinoma (HNC). We tested COX2 inhibitor, celecoxib in six types of HNC cells and analyzed the expression changes in proteins related to angiogenesis and apoptosis *in vitro*. We also evaluated proliferation, gelatinolysis and *in vitro* invasion. We used a hamster carcinogenesis model and a mouse tumorigenesis model for the *in vivo* evaluation of COX2 inhibition. We performed immunohistochemistry to assess changes in the expression of COX2, survivin and angiogenesis. Celecoxib administration caused decreases in the expressions of COX2, VEGF and survivin *in vitro*. Proliferation, *in vitro* invasion and gelatinolytic activity were reduced in HNC cell lines, but the effect was inconsistent across lines. COX2 inhibition retarded oral carcinogenesis from an early carcinogenic stage with increased apoptosis and decreased survivin expression. COX2 inhibition did not inhibit tumor growth, even with the COX2 downregulation and decrease in neovascularization. We conclude that COX2 inhibition has a chemopreventive effect, but its application as a treatment of HNC in a clinical setting still requires further research to overcome its limited anti-cancer effects.

INTRODUCTION

Head and neck carcinoma typically has a poor prognosis; the five-year survival rate of HNC is only 35-50%, despite recent advances in radiation therapy, improvements in surgical techniques, and the advent of aggressive chemotherapy protocols (Parkin et al., 2005). The poor prognosis of HNC may be explained by its aggressiveness, metastasis and tendency for local invasion (Woolgar et al., 2003).

Epidemiological studies have demonstrated the anticarcinogenic efficacy of non-steroidal anti-inflammatory drugs (NSAIDs) against a number of malignancies, and therefore many authors have proposed cyclooxygenase 2 (COX2) as a target for cancer prevention and treatment (Edwards et al., 2004; Park et al., 2004; Ristimaki et al., 2001; Ulrich et al., 2006; Yang et al., 2010). COX2 is a critical factor in carcinomas of various organs and plays a role in tumor development through enhancement of tumor cell survival (Chan et al., 1999; Jason et al., 2005; Ristimaki et al., 1997; 2001). Female transgenic mice that were designed to overexpress COX2 have high frequencies of mammary gland hyperplasia, dysplasia and metastatic tumors (Liu et al., 2001). In addition, overexpression of COX2 is frequently observed in premalignant tissues and cancers (Wilson et al., 1998; Zimmermann et al., 1999). COX2 influences several processes important to cancer development, such as apoptosis, angiogenesis and invasiveness, although the relative importance of each of these effects in tumorigenesis is uncertain (Chan, 2002; Dannenberg et al., 2001; Harris, 2007; Kulp et al., 2004). There is ample evidence suggesting that COX2 is mechanistically linked to the development of malignancies and that increased levels of COX2 activity can induce malignant transformation. Nevertheless, the practical clinical application of COX2 inhibition is still questionable and under study.

Although some studies have reported that COX2 inhibition shows antiangiogenic and chemopreventive effects on head and neck carcinomas (Crowell et al., 2006; Feng and Wang, 2006; Hao et al., 2009; Wang et al., 2002), the importance of COX2 in HNC is not fully understood. Goodin et al. claimed that it is necessary to continuously pursue the possibility of COX inhibition as a strategic treatment modality of HNC (Goodin and Shiff, 2004). Thus, the main purpose of our study was to investigate the feasibility of COX2 inhibition as a noble and strategic treatment modality for HNC. The main hypothesis is that direct and selective inhibition of COX2 using celecoxib could significantly alter the expression levels of several proteins related to apoptosis, invasion and angiogenesis in HNC *in vitro* and *in vivo*. As a result, COX2 inhibition should show strong anticarcinogenic, anti-proliferative and anti-invasive effects in HNC cell lines *in vitro*, and chemopreventive and anti-tumorigenic effects in animal models *in vivo*.

This study was composed of two parts. The first was to evaluate the changes in expression of some cancer markers in response to COX2 inhibition in several HNC cell lines and to

Department of Oral and Maxillofacial Surgery, School of Dentistry, Seoul National University, Seoul 110-749, Korea, [1]Dental Research Institute, Seoul National University, Seoul 110-749, Korea, [2]Department of Oral Oncology, National Cancer Center, Goyang 410-769, Korea
*Correspondence: myoungh@snu.ac.kr

Received September 25, 2009; revised November 11, 2009; accepted November 12, 2009; published online January 12, 2010

Keywords: angiogenesis, celecoxib, cyclooxygenase2 (COX2), head and neck carcinoma (HNC), invasion, oral squamous cell carcinoma

assess anti-cancer activity with respect to cytotoxicity and *in vitro* invasion. The second was to assess chemoprevention and anti-tumorigenesis effects *in vivo* in a hamster carcinogenesis model and a nude mouse tumorigenesis model.

MATERIALS AND METHODS

Cell culture and expression of COX2, survivin and VEGF in HNC cell lines

Six HNC cell lines, HSC-3, KB, SCC-4, SCC-9, SCC-15, and SCC-25 were purchased from the Japanese Cell Resources Bank (JCRB, Japan) and ATCC (American Type Culture Collection, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM: Gibco, USA) supplemented with 10% fetal bovine serum (Gibco), 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco) at 37°C in a humidified atmosphere of 5% CO_2. Cells were lysed in RIPA buffer containing protease and phosphatase inhibitors and equal amounts of protein extract were electrophoresed on 10% SDS polyacrylamide gels and electrotransferred to a nitrocellulose membrane, which was incubated in 5% BSA for 3 h followed by overnight incubation with antibodies against COX2 (rabbit polyclonal, Abcam, USA), survivin (sc-17779, Santa Cruz Biotechnology, USA) and VEGF (A-20, Santa Cruz Biotechnology). After washing the membrane, alkaline phosphatase-conjugated secondary antibodies were added and incubated. Color was visualized using ECL solution and quantified using a scanning densitometer. Protein extracts of human melanoma tissue were used for a positive control of COX2, VEGF and survivin.

The effect of COX2 inhibition on cell survival and invasiveness

The cytotoxic effects of celecoxib (Celebrex®, Pfizer, USA) were determined by MTT assay (Sigma, USA). Cells were seeded into 96-well plates at a density of 4×10^2 cells/well. The cells were serum-starved and then treated with various doses of celecoxib (0-50 μM) for 48 h. Cells were washed with Dulbecco's phosphate buffered saline (PBS; Sigma) and 0.5 mg/ml 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide was added. After incubation at 37°C in 5% CO_2 for 4 h, dimethyl sulfoxide (Sigma) was added and the absorbance at 570 nm was measured with a spectrophotometer.

For the *in vitro* invasion assay, matrigel matrix-coated 9-mm cell culture inserts (pore size: 8 μm; Becton Dickinson, USA) were set in a 24-well plate. Cells were seeded at a density of 5×10^3 cells/well into the upper layer of the culture insert and cultured with serum-free DMEM in the presence of 10 μM celecoxib for 24 h. Control cells were cultured in the same media without celecoxib. The lower layer of the culture insert contained 0.5 ml HT1080 cell conditioned media as a chemoattractant. After incubation, the cells remaining in the upper layer were swabbed with cotton and the cells penetrating into the lower layer were fixed with 5% glutaraldehyde and stained with hematoxylin. The total number of cells that passed through the Matrigel matrix and the 8 μm pores of the culture insert was counted using optical microscopy (× 100) and designated as the invasion index (cell number/area).

We performed gelatin zymography to evaluate the effect of COX2 inhibition on gelatinolytic activity. Gelatin was added to a 10% polyacrylamide separating gel to a final concentration of 0.1 mg/ml. In the celecoxib-treated group, cell-seeded plates were incubated with serum-free medium in the presence of 10 μM celecoxib for 24 h. Ten ml of the conditioned medium was filtered through a 0.22-μm filter and 12 μl medium was mixed with sample buffer (10% SDS, 50% glycerol, 25 mM Tris-HCl, pH 6.8, and 0.1% bromophenol blue) and loaded onto the gel. Following electrophoresis, the gels were washed twice in 2.5% Triton X-100 for 30 min at room temperature to remove the SDS. The gels were then incubated overnight at 37°C in a substrate buffer containing 50 mM Tris-HCl and 10 mM $CaCl_2$ at pH 8.0. The gel was stained with 0.15% Coomassie blue R250 in 50% methanol/10% glacial acetic acid for 20 min at room temperature and destained in the same solution without stain. Fresh serum-free medium containing an identical concentration of celecoxib was used as an internal negative control. The gelatinolytic activity of activated (62 kDa) forms of MMP-2, visible as a band on the gel, was measured by two-dimensional densitometric analysis using the BioRad scanning densitometer. The activity of the activated form of MMP-2 present in 10 μl (0.5 μg total protein) of conditioned media from primary cultured normal oral mucosal fibroblasts was defined as 10 arbitrary units of MMP-2.

Evaluation of chemopreventive effect and apoptosis

Male Syrian hamsters aged 8-10 weeks and weighing between 110 and 125 g were obtained and maintained under controlled conditions of temperature and humidity with an alternating light/dark cycle. Animals were housed four to a polypropylene cage and were provided with a standard pellet diet and water *ad libitum*. A total of 80 hamsters were divided into two groups, a control group without celecoxib treatment and an experimental group with celecoxib treatment. Both groups were further divided into four subgroups: four weeks (n = 10), eight weeks (n = 10), 12 weeks (n = 10) and 16 weeks (n = 10). All animals were painted with 0.5% DMBA on their left buccal pouches once a day until the end of experiment. Animals in the experimental group were given celecoxib orally at a dose of 1500 ppm (Feng and Wang, 2006; Wang et al., 2002), starting one week before exposure to the carcinogen and continuing until sacrifice of the animals. The experiment was terminated after 16 weeks and all animals were sacrificed by cervical dislocation. The frequency of tumor bearing hamster were obtained and the number of tumor mass per animal were counted and compared.

For histopathological examination, buccal pouch tissues were fixed in 10% formalin and embedded in paraffin, and then 4-5 μm sections were cut and saved for H&E staining and immunohistochemical analysis. Induction of apoptosis in hamster carcinoma cells was confirmed using a TUNEL system kit (Takara, Japan). After deparaffinization, slides were washed with distilled water, incubated with 20 μg/ml Proteinase K at room temperature for 15-30 min, and washed with PBS. Slides were incubated in 3% H_2O_2 for 5 min to inactivate endogenous peroxidase and washed. After the addition of 50 μl labeling reaction mixture and incubation in a 37°C humidified incubator for 60-90 min, the reaction was terminated by washing in three changes of PBS for 5 min each wash. Slides were incubated in 70 ml anti-HRP conjugate at 37°C for 30 min, and washed three times in PBS for 5 min per wash. After staining with DAB at room temperature for 10-15 min, the reaction was terminated by washing with distilled water. The apoptosis index was defined as the percentage of positive cells in 1,000 cells.

Evaluation of anti-tumorigenesis

We chose KB and SCC-9 cell lines for inoculation of nude mice because they showed a significant difference in COX2 inhibition *in vitro* and possess celecoxib-sensitive and resistant properties in proliferating assays, and both cell lines were tumorigenic in the preliminary study. Detached cells were diluted and emulsified with medium to a final cell concentration of 5×10^7cells/ml. Two hundred μl of the emulsion was inoculated subcutaneously into the right flank of thirteen six-to-eight-week-old female BALB/c

Table 1. Change of *in vitro* invasion and gelatinolytic activity by COX2 inhibition

		KB	HSC3	SCC-4	SCC-9	SCC-15	SCC-25
Migrating cell count (cells/area)	Control	155.1 ± 11.7	142.1 ± 11.5	368.2 ± 9.7	68.2 ± 8.4	102.7 ± 19.5	182.4 ± 8.1
	Celecoxib treated	86.0 ± 5.3*	45.2 ± 6.4*	105.5 ± 9.8*	72.5 ± 4.1	99.8 ± 4.4	191.5 ± 6.9
Gelatinolytic activity (units/μg)	Control	66.7 ± 4.4	76.4 ± 3.7	55.1 ± 4.9	53.9 ± 9.2	87.5 ± 7.0	22.4 ± 4.2
	Celecoxib treated	49.9 ± 2.7*	69.8 ± 7.9*	42.3±4.0*	7.1 ± 2.1*	91.9 ± 10.7	11.9 ± 3.1*

*Significantly lower than the control group ($p < 0.05$)

nude mice (Samtako, Korea) weighing approximately 20 g which were kept in an environment complying with NIH guidelines for the care and use of laboratory animals. Each group contained ten animals and celecoxib was administered when the volume of the ensuing mass reached 75-100 mm^3. Animals in the experimental group were given celecoxib orally at a dose of 1,500 ppm every day until sacrifice. The lengths and widths of the masses were measured by caliper every three days and the tumor volumes (in cubic millimeters) was calculated using the following equation: Volume (mm^3) = Length × $Width^2$ × 1/2. The relative tumor volume (RTV) was calculated as RTV = Vi/Vo, where Vi is the tumor volume at any given time and Vo is the volume at the time of initial treatment. Each animal was weighed twice a week to evaluate any drug toxicity effects. The mice were sacrificed 30 days after initial treatment and the tumor mass was excised and frozen for immunohistochemistry.

Immunohistochemical evaluation of COX2, survivin, VEGF and CD31

Immunohistochemical analysis of survivin and COX2 expression were performed using rabbit polyclonal survivin (ab469, Abchem, UK) and COX2 (Cayman Chemical, USA) antibodies, according to previously published methods (Crowell et al., 2006; Hsue et al., 2008). COX2 immunoreactivity in the hamster model was graded according to the most carcinogenic lesion present in the buccal pouch, on a point intensity scale from 0 to 2 (0 = absent/weak; 1 = intermediate; 2 = intense staining) by two oral pathologists. Survivin immunoreactivity was measured using the method previously described by Kennedy et al (Kennedy et al., 2003). Survivin immunoreactivity was semiquantitatively evaluated based on the percentage of cells demonstrating distinct nuclear and/or diffuse cytoplasmic immunohistochemical reactivity. Five areas were chosen for analysis of each slide at × 40 magnification and nuclear and cytoplasmic tumor cell immunoreactivities were not separately assessed. Arbitrary scores for immunoreactivity were assigned based on the percentage of positive cell numbers/observed cell numbers as follows: grade 0, < 5%; grade 1, 5-20%; grade 2, 21-50%; grade 3, 51-75%; grade 4, > 76%. A cutoff value of > 20% was established as strong expression and tumors with a score of 0 or 1 were considered to have weak expression.

For assessment of angiogenesis in the nude mouse model, we used JC70 monoclonal antibody (DAKO) recognizing CD31 (platelet/endothelial cell adhesion molecule; PECAM-1, 1:20). Blood vessel density was assessed using the method described by Bosari (Bosari, 1992). Briefly, the three areas with the highest vascularization were chosen at low power (× 40 and 100). The number of microvessels in each area was counted under high magnification (× 200) and the average value of the three fields was used to evaluate the relationship between survivin expression and microvessel density. Microvessels adjacent to necrotic areas were excluded from the analysis.

Statistical analysis

All *in vitro* experiments were repeated at least three times until reproducible results were obtained. The statistical significance of the differences between the two groups was determined using a Mann-Whitney U test or a *t*-test. A *p* value of < 0.05 was regarded as significant.

RESULTS

The effects of COX2 inhibition on expression of COX2, survivin, and VEGF and *in vitro* cytotoxicity

After immunoblotting, all cell lines showed strong expression of COX2 protein, but the level of expression varied between cell lines (Fig. 1A). The COX2/β-actin relative intensity was significantly higher in KB, SCC9, SCC15, and SCC25 than in HSC3 and SCC4 cell lines. Celecoxib administration (10 μM) significantly inhibited COX2 expression in all HNC cell lines, but the level of inhibition also varied according to each cell line (Fig. 1B). SCC4 cells showed no detectable expression and KB cells showed low expression with celecoxib treatment, whereas other cell lines still notably expressed COX2 protein in spite of inhibition. In the KB and SCC4 cell lines, the difference in COX expression before and after celecoxib administration was significantly higher than that of other cell lines. COX2 expression in the KB and SCC4 post-treatment group decreased by 87.6% and 99.9% of expression before treatment, respectively, while other cell lines expressed levels of COX2 over 50% of the original expression. COX2 inhibition also caused significant reduction in survivin and VEGF expression in all HNC cell lines (Fig. 1E). In cytotoxicity assays, cells were exposed to continuous celecoxib treatment at concentrations of 0, 10, 20, 30, 40 and 50 μM. Inhibition of COX2 induced dose-dependent cytotoxicity only in KB and SCC4 cell lines, but had no significant anti-proliferative effect on HSC3, SCC9, SCC15 and SCC25 cell lines (Fig. 2). In the two sensitive cell lines (KB and SCC-4), 30 μM celecoxib reduced cell growth by 50%, whereas this concentration of celecoxib had no significant inhibitory effect on cell proliferation of the other cell lines.

Effects of COX2 inhibition on invasiveness *in vitro*

Celecoxib significantly reduced the invasive potential of KB, HSC-3 and SCC-4 cells (Table 1). In an *in vitro* invasion assay, COX2 inhibition reduced the degree of cellular invasion through the artificial basement membrane as shown in Fig. 3 (HSC-3 case). The invading cell counts for celecoxib-treated KB, HSC3 and SCC4 cells were 86.0 (± 5.3), 45.2 (± 6.4) and 105.5 (± 9.8), respectively, compared with 155.1 (± 11.7), 142.1(± 11.5) and 368.2 (± 9.7) for untreated controls ($p < 0.05$). Meanwhile, when we performed gelatin zymography, there were significant differences in the gelatinolytic activity by MMP-2 activation between treated and control cells for all cell lines except the SCC15 cell line shown in Fig. 3 and Table 1. For example, in the KB cell line, the MMP-2-induced gelatinolytic activity of the celecoxib-treated group was 66.7 (± 4.4) U, and that of the

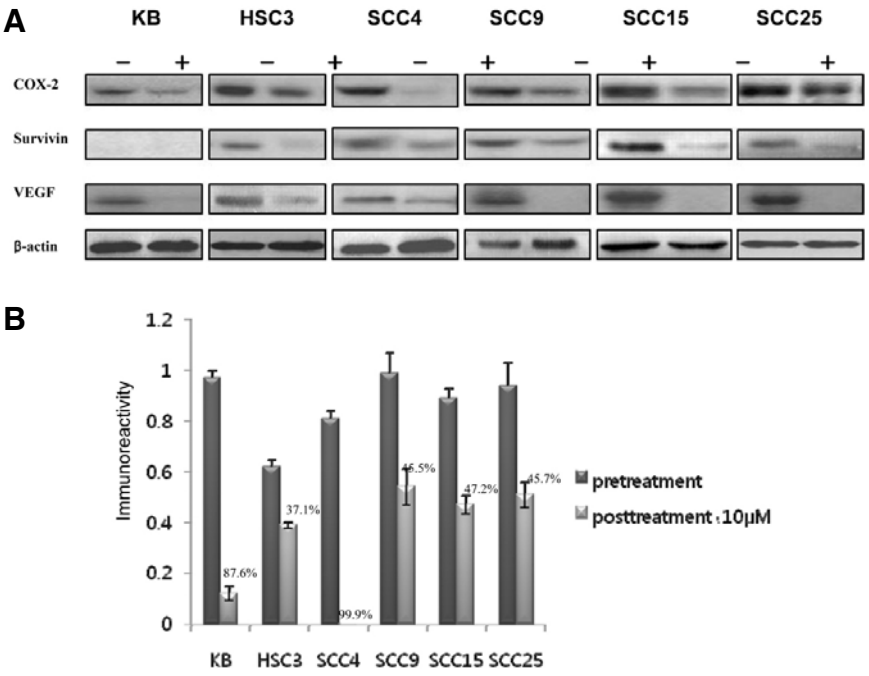

Fig. 1. Expression of COX2, survivin and VEGF in HNC cell lines. (A) Immunoblotting results for COX2, survivin, and VEGF proteins in six kinds of HNC cell lines before and after treatment (10 μM, 24 h). Each protein expression varied according to each HNC cell line. (-: pretreatment, +: posttreatment). (B) COX2 inhibition with celecoxib. COX2 inhibition with celecoxib also varied significantly depending on cell line. COX2 was inhibited by 87.6% and 99.9% in KB and SCC4 cells, respectively but not by 50% in other cell lines. With COX2 inhibition, expression of survivin and VEGF were also significantly inhibited ($p = 0.011$).

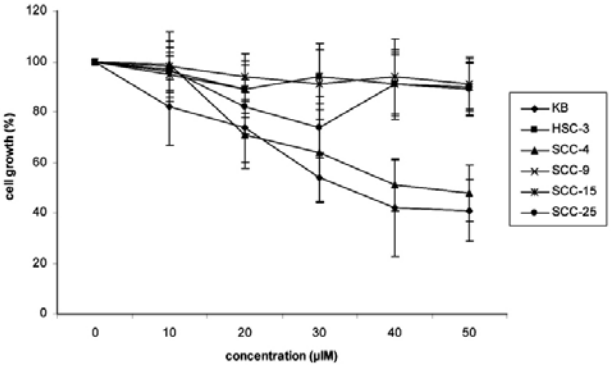

Fig. 2. Cytotoxic effects of COX2 inhibition in six types of HNC cell lines. The anti-proliferating effects of COX2 inhibition were evaluated by MTT assay. Cells were treated with 0-50 μM celecoxib for 72 h and the percentage of growth inhibition was determined after 48 h of treatment. KB and SCC-4 cell lines had significant dose-dependent growth inhibition due to COX2 inhibition ($p = 0.007$), but no effect was observed in HSC3, SCC9, SCC15 and SCC25 cell lines ($p > 0.05$).

control group decreased to 49.9 (± 2.7)U ($p < 0.05$).

Chemopreventive effects of celecoxib in animal models

In the hamster buccal pouch carcinogenesis model, we observed cancer phenotypes of hyperkeratosis, mitotic figures and nuclear pleomorphism in the control group. At the end of the experiment, all animals in the control group and eight of ten animals in the celecoxib treated group exhibited multiple tumors and all animals demonstrated histologically carcinomatous figures (Table 2). The number of tumor mass per animal was higher in the control group (2.7 ± 0.78) than in the group of hamsters that received celecoxib (1.7 ± 1.42), however, there's no significance. However, in histological evaluation, COX2 inhibition also caused delays in the early stages of carcinogenesis; at weeks 8 and 16, the levels of hyperkeratosis, mitotic figures and nuclear pleomorphism were lower in the celecoxib-treated group than in the control group (Table 2). The progress of carcinogenesis was inhibited by celecoxib administration until 16 weeks, when we observed significant histological differences between celecoxib-treated and control animals. In addition, the celecoxib-treated group showed higher apoptosis indexes than the control group at eight, 12 and 16 weeks, and there was a statistically significant difference in the degree of apoptosis between the experimental and control groups (Figs. 4A and 4B, Table 2).

Immunohistochemical analyses revealed that the levels of COX2 inhibition in oral carcinogenesis were significant (Figs. 4C and 4D). While the average rank of COX2 expression in the control group was 13.25 to 14.40, COX2 expression significantly decreased to 7.25 to 7.60 in the drug administered group (Table 2, $p < 0.05$). Survivin immunoreactivity was primarily observed in the cytoplasm of carcinogenic cells. Survivin was detected in the early stages of carcinogenesis at four weeks until termination of the experiment at 16 weeks. The immunoreactivity of survivin expression became stronger as carcinogenesis progressed in the control group (Figs. 4E and 4F, Table 2). With COX2 inhibition, the immunoreactivity of survivin, also decreased in oral carcinogenesis process, as shown in Table 2 and Figs. 4E and 4F. With regard to the intensity of survivin expression, it was more definite and extensive in the control group, compared to the sparse and weak expression in the treated group. We observed five cases of strong survivin expression the 12-week control group and seven in the 16-week control group. On the contrary, we observed two cases of strong survivin expression in the 12-week experimental group and two cases in the 16-week experimental group (Table 2).

Anti-tumorigenic effects of celecoxib in animal models

In the anti-tumorigenesis experiment using nude mice, there were no significant differences in body weight between the control and experimental groups during the course of the experiment. The relative tumor volumes (RTV) of two kinds (KB and

Table 2. Chemopreventive effect of COX2 inhibition in oral carcinogenesis model

	Control group				Celecoxib treated group			
	4 w	8 w	12 w	16 w	4 w	8 w	12 w	16 w
Hyperkeratosis	+	++	+++	+++	±†	+†	++†	++†
Acanthosis	±	+	+++	+++	-†	+	++†	++†
Mitotic figures	-	+	+++	+++	-	-†	++†	++†
Nuclear Pleomorphism	-	+	++	+++	-	-	+†	+†
Apoptotic index	17.4 (± 3.8)	13.2 (± 4.6)	12.1 (± 3.2)	12.7 (± 3.0)	16.5 (± 7.2)	24.1 (± 5.4)*	23.7 (± 7.7)*	25.8 (± 9.1)*
COX2 immunoreactivity††	13.40	13.50	13.25	14.40	7.60**	7.50**	7.25**	7.60**
Survivin immunoreactivity‡	2/10	3/10	5/10	7/10	1/10	1/10	2/10**	2/10**
Frequency of tumorigenesis	0/10	0/10	4/10	10/10	0/10	0/10	3/10	8/10
Number of tumor per animal	0	0	0.5 (± 0.67)	3.1 (± 1.30)	0	0	0.4 (± 0.66)	1.7 (± 1.42)

w, week; -, negative; ±, trace; +,mild; ++, moderate; +++, marked
*, significantly higher than the control group ($p < 0.05$)
†, significant weaker than the control group ($p < 0.05$)
††, average rank in Mann-Whitney U test
**, significantly weaker expression of COX2/survivin than the control group ($p < 0.05$)
‡, case number of strong survivin expression/total case number

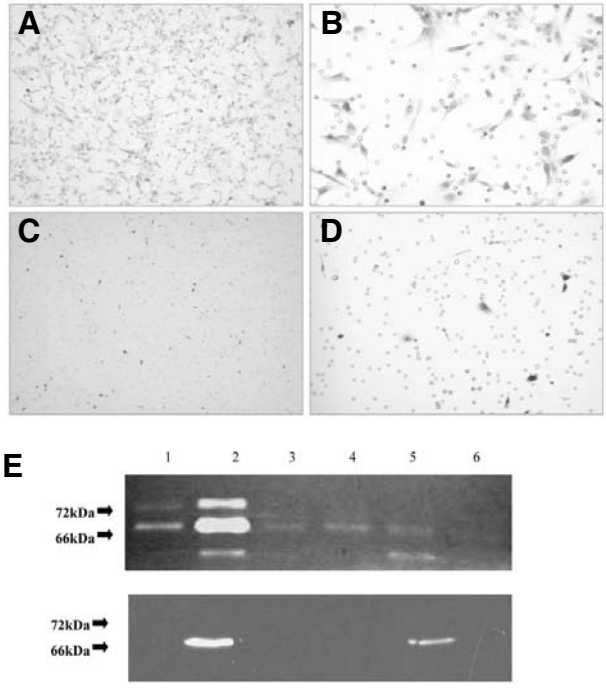

Fig. 3. *In vitro* invasion assay in HSC3 cell lines (A-D) and gelatin zymography (E). In the celecoxib-treated HSC-3 cell lines (10 μM, 24 h), COX2 inhibition significantly reduced the invasiveness. In (A, B) (control, × 100 and × 200, respectively) and in (C, D) (celecoxib-treated, × 100 and × 200, respectively), celecoxib-treated HSC-3 cells demonstrated a significant decrease in the number of cells penetrating and migrating through the artificial cell membrane. Gelatin zymography reveals the gelatinolytic activities of the activated (62 kDa) forms of MMP-2 as white banding. A significant difference in the gelatinolytic activity is detected between the control group and the experimental group in KB, HSC3, SCC4, SCC9 and SCC25 cell lines (lane 1, KB; lane 2, HSC3; lane 3, SCC4; lane 4, SCC9; lane 5, SCC-15; lane 6, SCC-25).

of SCC-9) of HNCs increased during the experiment, as shown in Fig. 5. For example, on the 30th day after inoculation, the RTV for KB cells was 9.2 (± 4.1) in the control group and 7.8 (± 3.2) in the celecoxib-treated group. There were no statistically significant differences between the RTVs of the celecoxib-treated and control groups during the course of the experiment, indicating that celecoxib treatment had no effect on the growth of xenotransplanted HNC tumors in nude mice. Likewise, in SCC-9-bearing nude mice, the growth rates of tumor masses were not significantly different between the celecoxib-treated and control groups at any time during the experiment.

Meanwhile, an immunohistochemical study showed that COX2 inhibition was significant in celecoxib-treated tumors as shown in Figs. 6A and 6B. COX2 reactivities in the control groups for KB cell tumors and SCC9 cell tumors were 14.0 and 13.75, respectively. In the treated groups, COX2 reactivity significantly decreased to 7.0 (KB cells) and 7.25 (SCC9). COX2 inhibition affected survivin expression of both cell tumors. On the contrary, survivin expression in a xenotransplanted tumor was not significantly affected by COX2 inhibition. In the control group, nine of 10 KB and eight of 10 SCC9 showed strong survivin expression. Likewise, eight of 10 animals in both cell tumors of the treated group were categorized as having strong survivin expression. A statistically significant lower microvessel density was observed in the celecoxib-treated group compared to the control group (Figs. 6E and 6F). Microvessel densities of the control group were 18.90 (± 5.19) in KB and 18.67 (± 4.89) in SCC9, but those of the treated group scored 14.10 (± 4.71) in KB and 15.60 (± 5.60) in SCC9. The differences in microvessel density between the control and experimental groups were significant for both cell type tumors ($p < 0.05$).

DISCUSSION

Although transcription of COX2 is constitutively up-regulated in several types of malignancies, including oral cancer (Jason et al., 2005; Tang et al., 2003), one of the major concerns arising from the present study is the uncertainty regarding the anticancer effects of COX2 inhibition on various HNC cell lines, in

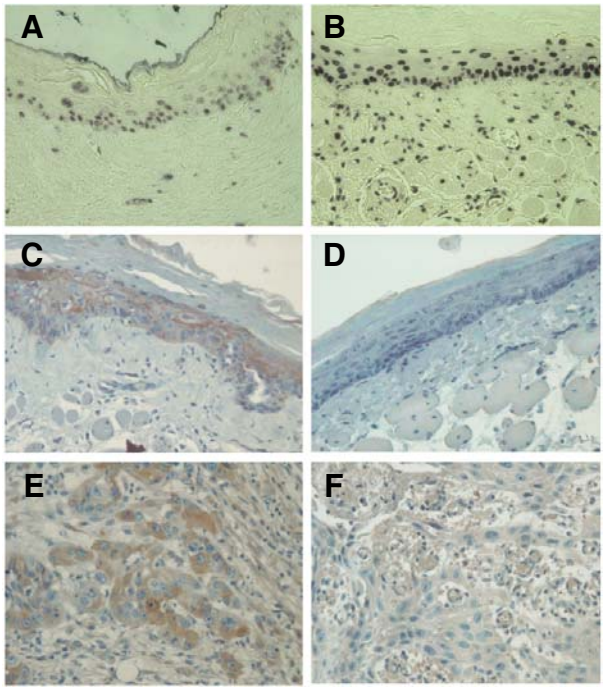

Fig. 4. Chemopreventive effects in a hamster buccal pouch carcinogenesis model. The eight-week 1500 ppm of celecoxib treated hamster buccal pouch epithelium (B) had a significantly higher apoptotic index than the control (A). COX2 inhibition was significant throughout the entire experimental period as shown in (C) (eight-week control group) and (D) (eight-week experimental group). Survivin expression in the eight-week drug-treated group (E) was also lower than that in the eight-week control group (F) (original magnification of all figures, × 100).

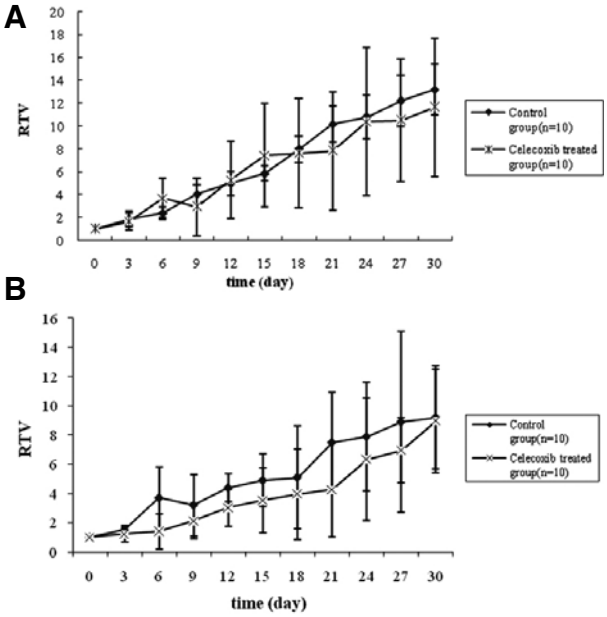

Fig. 5. Anti-tumorigenic effects of COX2 inhibition. As shown in (A, B) (KB and SCC-9 cell line tumorigenesis, respectively), the relative tumor volume (RTV) increased throughout the experimental period in both groups. Note that the RTV in the 1,500 ppm of celecoxib-treated group is lower than that of the control group, but the difference is not statistically significant ($p > 0.05$).

which the expression level of COX2 is still unknown. Immunoblot analysis showed that all of the HNC cell lines had strong COX2 expression, although the amount of expression varied between cell lines. In addition, each HNC cell line showed a different sensitivity to celecoxib treatment. In particular, KB and SCC4 cell lines showed an 87.6% and 99.9% decrease in survivin expression after celecoxib treatment, while the amount of COX2 downregulation did not reach 50% of the original expression in other cell lines as shown Fig. 1. The inducibility of COX2 can be explained, at least in part, by the presence of numerous cis-acting elements in the 5'-flanking region of the COX gene, such as NF-kB, NF-IL6 and CRE sites (Inoue et al., 1995; Lerebours et al., 2008) and by the increased stability of COX2 mRNA (Sheng et al., 2000). In addition, numerous studies have reported that the anti-cancer effect of celecoxib is mediated by both COX2-dependent and COX2-independent mechanisms (Haupt et al., 2006; Lai et al., 2003; Maier et al., 2004). Thus, the present *in vitro* data from this study highlight the variable inducibility of COX2 in HNCs and the unpredictability of COX2 inhibition by celecoxib.

Survivin is a member of the inhibitor of apoptosis (IAP) family, whose members have been shown to inhibit activated caspases (Shin et al., 2001). Recently, the focus of survivin research has shifted towards survivin as a target of cancer therapy, especially since studies have revealed that the role of survivin in cancer cells is not limited to apoptosis inhibition, but may also be associated with aggressive characteristics of cancer such as angiogenesis and closely associated with invasive potential (Duffy et al., 2007; Ouhit et al., 2007). Numerous studies have demonstrated the close relationships between survivin and COX2 and reported its clinical implications in colon, endometrial and breast carcinoma (Barnes et al., 2006; Erkanli et al., 2007; Mori et al., 2007; Pyrko et al., 2006). Survivin is reported to be a significant tumor marker in oral carcinogenesis (Hsue et al., 2008). In addition, survivin is a death encounter as well as a survival protein for invading tumor cells (Ouhit et al., 2007). Thus, we also focused on the change of survivin expression with COX2 inhibition in the present study. In western blot analyses, most HNC cell lines, except for the KB cell line, strongly expressed survivin and celecoxib significantly downregulated survivin expression in all studied HNC cell lines in spite of the varying levels of COX2 inhibition. In addition, our western blot results showed a change in VEGF expression in HNC before and after COX2 inhibition. It has been well documented that the growth of solid tumors and the formation of metastases are dependent on angiogenesis, and in the case of head and neck carcinomas, VEGF plays a critical role in carcinogenesis, tumorigenesis and progression (Johnstone and Logan, 2006). In the stable state, VEGF was expressed in the six types of HNC cell lines tested in the present study (Fig. 1). COX2 inhibition also gave rise to a significant decrease in VEGF expression after administration of celecoxib. Accordingly, numerous previous reports and the Western blot results of this study imply that COX2 inhibition may cause additional anti-cancer effects related to survivin and VEGF in HNCs. However, it remains questionable whether COX2 inhibition is a practical treatment modality for HNC considering the subsequent *in vitro and in vivo* results of this study. In a proliferation assay, celecoxib showed a dose-dependent, anti-proliferation effect in only two HNC cell lines, KB and SCC4 (Fig. 2). COX2 inhibition with celecoxib had no effects on proliferation in the other four HNC cell lines. A previous study indicated that COX2 inhibition is associated with

Table 3. Anti-tumorigenesis effect of COX2 inhibition in nude mice model

	KB		SCC9	
	Control group	Celecoxib treated group	Control group	Celecoxib treated group
COX2 immunoreactivity (average rank in Mann-Whitney U test)	14.0	7.0*	13.75	7.25*
Survivin immunoreactivity (case number of strong survivin expression/ total case number)	9/10	8/10	8/10	8/10
Microvessel density average (± SD)	18.90 (± 2.19)	14.10 (± 2.71) *	18.67 (± 2.89)	15.60 (± 2.60) *

*, $p < 0.05$

only a minor effect on cell growth at low concentrations (2-5 μM, Dvory-Sobol et al., 2006). The present study also showed that the concentration required to induce > 50% growth inhibition (30 μM) was more than two-fold higher than that required to inhibit COX2 activity (10-15 μM) even in the two cell lines with decreased proliferating activity. The significant gap between the physiologically achievable concentration and the concentration required for the anti-cancer effect may be a serious barrier to the clinical application of this drug. Meanwhile, an interesting observation of this study is that two sensitive cell lines both showed remarkable downregulation of COX2 compared to other cell lines (Fig. 1). In particular, these two cell lines also had decreased *in vitro* invasion and gelatinolytic activity with COX2 inhibition, in contrast to other cell lines, which showed variable results (Fig. 3, Table 1). In addition, both celecoxib-sensitive cell lines expressed COX2 as strongly as other resistant HNC cell lines. Thus, the expression level of COX2 in the stable stage does not seem to be a predictive factor of celecoxib sensitivity in HNC. These results might imply anticancer effect of COX2 inhibition depends on the level of COX2 inhibition with a certain COX2 inhibitor in a certain HNC rather than its original expression level of COX2.

In addition to direct cytotoxicity, another main interest was the anti-invasive activity of celecoxib in this study because tumor invasion and subsequent metastasis is a major problem in the clinical management of malignancies. In a previous report examining the effects of COX2 inhibition on the invasiveness of two HNC cell lines (NA and HSC-4), NS-398, a highly selective inhibitor of COX2, inhibited invasiveness as determined by the same Matrigel invasion assay used in this study (Kinugasa et al., 2004). Likewise, there are several reports that COX2 inhibitors suppress the invasiveness of various cancer cells (Tang et al., 2003; Yamazaki et al., 2002; Yang et al., 2003), although the mechanism for this is not clear. Accordingly, we presumed that COX2 inhibition would decrease the ability of cells to penetrate through an artificial cell membrane in our experimental setting and that this decreased invasiveness was the result of inhibition of a major proteinase, MMP-2. We focused on MMP-2 because several reports have indicated a correlation between MMP-2 activity and metastatic potential in various cancer cells (Bernardo et al., 2003; Yang et al., 2003). Figure 3 shows that celecoxib treatment affects the *in vitro* invasive properties of three cell lines (KB, HSC3, and SCC4) and gelatinolytic activities of four cell lines (KB, HSC3, SCC4 and SCC9). These data suggest that inhibition of COX2 may affect the invasive characteristics of HNC cells by decreasing gelatinolytic activity, without cellular cytotoxicity. However, there was no decrease in *in vitro* invasion and gelatinolytic activity in two HNC cell lines (SCC15 and SCC25). In addition, in the case of SCC9 cells, direct invasion was not inhibited, although gelatinolytic activity was significantly reduced by celecoxib (Fig. 3, Table1). Given that the invasive property of a certain HNC is not always dependent on MMP-2 activation and that the mechanism underlying the anti-invasive effect of NSAIDs is variable (Attiga et al., 2000), our data demonstrate that direct inhibition of invasiveness by celecoxib is limited and varies depending on HNC cell type.

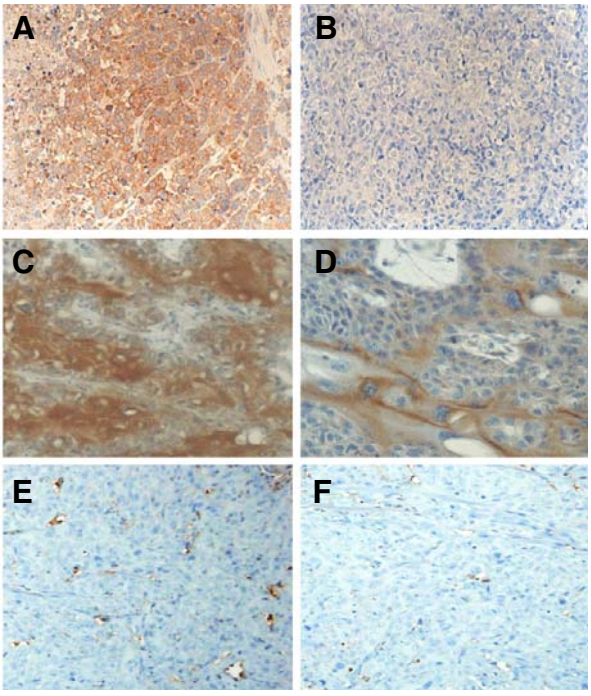

Fig. 6. Anti-tumorigenic effects in COX2 inhibition (nude mouse tumorigenesis model). Celecoxib treatment (1,500 ppm a day) caused a significant reduction in COX2 expression in an oral squamous cell carcinoma cell xenografted model [(A, B), original magnification × 100]. However, survivin expression was not significantly reduced, even with the COX2 inhibition [(C, D), original magnification × 200]. Meanwhile, microvessel density decreased significantly in the drug-treated group [(E) and (F), original magnification × 100] [(A) COX2 expression in control group of KB cell tumor, (B) COX2 expression in drug treated group of KB cell tumor, (C) survivin expression in control group of SCC9 cell tumor, (D) survivin expression in drug treated group of SCC9 cell tumor, (E) CD31 in control group of SCC9 cell tumor, (F) CD31 in drug treated group of SCC9 cell tumor].

Chemoprevention involves blocking the DNA damage that initiates carcinogenesis, or arresting or reversing the progression of premalignant cells in which such damage has already

occurred. Recent advances in our understanding of what causes cancer and the consequent ability to provide genetic assessment of susceptibility have led to the need to find agents that can effectively reverse, stop, or at least delay the carcinogenic process. Chronic inflammation plays an important role in carcinogenesis and tumorigenesis of solid tumors, including HNC (Le Bitoux et al., 2008; Maeda and Omata, 2008). Inflammatory mediators such as cytokines, eicosanoids and growth factors are thought to play a critical role in the initiation and maintenance of cancer cell survival and growth. Over-expression of COX2 in precancerous lesions leads to increased levels of etheno-adducts, possibly as a result of increased oxidative stress (Bartsch, 2000; Bartsch and Nair, 2000). In humans, treatment with 400 mg celecoxib twice daily for six months was found to significantly reduce the number of polyps in familial adenomatous polyposis (FAP) patients (Steinbach et al., 2000). The hamster carcinogenesis model used in this study shows that COX2 inhibition definitely exerts a chemopreventive effect from an early carcinogenesis stage (Table 2). Although there was no statistical significance in the frequency of tumor and the number of tumorigenesis, as carcinogenesis proceeds, celecoxib effectively delays the onset and slows the growth of a DMBA-induced oral tumor in hamster cheek pouches, with increased apoptosis (Figs. 4A and 4B). Our immunohistochemical analysis revealed that COX2 inhibition was significant throughout the experimental period (Figs. 4C and 4D, Table 2). In addition, there was a significant difference between control animals and experimental animals in survivin expression as shown in Figs. 4E and 4F. At 12 and 16 weeks, the treated animals showed weaker expression of survivin than control animals. These results imply that a clone of premalignant cells switches on re-expression of survivin when it acquires the cancer phenotype and changes into a malignant cell by genetic alteration during oral carcinogenesis, and that COX2 inhibition downregulates survivin expression and subsequently delays oral carcinogenesis. Although the exact mechanism of cellular signaling between two proteins is still unclear, we conclude that COX2 inhibition has a definite effect on oral carcinogenesis and this activity is mediated by the increased apoptosis caused by downregulation of survivin.

Based on our *in vitro* and hamster model studies, we postulate that COX2 inhibition by celecoxib exhibits effective anticancer activity via a direct cellular effect as well as a chemopreventive effect. Nonetheless, it is still questionable in the effective value of COX2 inhibition as a major anti-cancer agent for HNC because it failed to inhibit tumor growth in this study. As shown in Fig. 5, COX2 inhibition showed no effect on tumorigenesis in two types of xenotransplanted nude mouse models; the tumor mass generated by inoculation with KB or SCC-9 cells grew without any reduction in mass size throughout the experimental period despite COX2 inhibition (Fig. 6B). We originally hypothesized that, at the very least, tumor mass growth of the KB tumor would be reduced because growth of KB cells is inhibited by COX2 inhibition *in vitro*. However, there was no evidence of growth inhibition in either tumor mass following COX2 inhibition. There is a report that cell lines that do not overexpress functional cyclooxygenase-2 are resistant to the normally achievable plasma levels of celecoxib (Haupt et al., 2006). However, both cell tumor types observed in this study showed strong expression of COX2 *in vitro* and *in vivo* (Figs. 1 and 6A) and immunohistochemical observation revealed significant downregulation of COX2 in a xenotransplanted tumor of both cell types (Fig. 6B). Moreover, another report using the same KB cell line and the same dose of celecoxib as this study demonstrated a significant delay in tumor growth. The only difference between the previous report and ours was in the initiation of drug administration; the administration started on the same day as cell inoculation in the previous study while administration started after observation of tumorigenesis in this study (Yang et al., 2010). In addition, survivin expression in the drug-treated tumor group was not significantly affected in spite of significant inhibition of COX2 (Fig. 6B, Table 3), while survivin expression was remarkably diminished in the hamster carcinogenesis model of this study. These results demonstrate that COX2 inhibition is not effective against tumor progression once tumorigenesis is initiated and an observable tumor mass is formed as Mohan et al. also reported that COX 2 overexpression is primarily related to an early stage of carcinogenesis (Mohan and Epsteina, 2003). All of the above results suggest that COX2 inhibition has a differential anti-cancer effect on carcinogenesis and tumorigenesis in HNC and that effective anticancer action can be achieved at an early stage of oral carcinogenesis and tumorigenesis, although the exact mechanism needs further investigation.

Angiogenesis is essential for cancerous tissues to receive adequate nutrition for their continuous growth, and tumors exhibiting high numbers of microvessels have been shown to possess high metastatic potential (Folkman, 1990; Folkman and Shing, 1992). Previous studies have shown that celecoxib-induced COX2 inhibition has an anti-angiogenic effect on oral carcinogenesis and cancer progression (Mohan and Epsteina, 2003; Wang et al., 2002). Likewise, COX2 inhibition also causes downregulation of VEGF *in vitro* (Fig. 1) and a significant decrease in microvessel density in the nude mouse tumorigenesis model (Figs. 6E and 6F) in the present study. However, as presented earlier, tumor growth of both cell types was not inhibited throughout the experimental period. Given that the mechanism of angiogenesis is multifaceted and depends on the cancer type, these results definitely imply that anti-neovascularization involving PGE2 and VEGF is not sufficient to inhibit tumor cell growth in HNC. These results might reflect high biological redundancy and low dependence on angiogenesis, which is due to the genetic specificity arising from high vascularity of the head and neck areas (Gleich et al., 1998).

We conclude that the inducibility of COX2 and the direct cytotoxic mechanism using celecoxib is not predictable and may vary according to type of cancer. Accordingly, the anticancer strategy using COX2 inhibition may also be variable in a clinical setting. Nonetheless, COX2 inhibition in HNC warrants further research because of its stable anti-carcinogenic activity and its safety as a combined drug. In addition, further studies will provide valuable information on the role of COX2 in HNC in terms of its multifaceted mechanisms involving both enzyme dependent and independent pathways.

ACKNOWLEDGMENT

This research was supported by Basic Science Research Program Through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2009-0075293).

REFERENCES

Attiga, F.A., Fernandez, P.M., Weeraratna, A.T., Manyak, M.J., and Patierno, S.R. (2000). Inhibitors of prostaglandin synthesis inhibit human prostate tumor cell invasiveness and reduce the release of matrix metalloproteinases. Cancer Res. *60*, 4629-4637.

Barnes, N., Haywood, P., Flint, P., Knox, W.F., and Bundred, N.J. (2006). Survivin expression in *in situ* and invasive breast cancer relates to COX-2 expression and DCIS recurrence. Brit. J. Cancer *94*, 253-258.

Bartsch, H. (2000). Studies on biomarkers in cancer etiology and prevention: a summary and challenge of 20 years of interdisciplinary research. Mutat. Res. *462*, 255-279.
Bartsch, H., and Nair, J. (2000). Ultrasensitive and specific detection methods for exocylic DNA adducts: markers for lipid peroxidation and oxidative stress. Toxicology *153*, 105-114.
Bernardo, M.M., and Fridman, R. (2003). TIMP-2 (tissue inhibitor of metalloproteinase-2) regulates MMP-2 (matrix metalloproteinase-2) activity in the extracellular environment after pro-MMP-2 activation by MT1 (membrane type 1)-MMP. Biochem. J. *374*, 739-745.
Bosari, S. (1992). Microvessel quantitation and prognosis in invasive breast carcinoma, Hum. Pathol. *23*, 755-761.
Chan, T.A. (2002). Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. Lancet. Oncol. *3*, 166-174.
Chan, G., Boyle, J.O., Yang, E.K., Zhang, F., Sacks, P.G., Shah, J.P., Edelstein, D., Soslow, R.A., Koki, A.T., Woerner, B.M., et al. (1999). Cyclooxygenase-2 expression is up-regulated in squamous cell carcinoma of the head and neck. Cancer Res. *59*, 991-994.
Crowell, P.L., Schmidt, C.M., Yip-Schneider, M.T., Savage, J.J., Hertzler, II D.A., and Cummings, W.O. (2006). Cyclooxygenase-2 expression in hamster and human pancreatic neoplasia. Neoplasia *8*, 437-445.
Dannenberg, A.J., Altorki, N.K., Boyle, J.O., Dang, C., Howe, L.R., Weksler, B.B., and Subbaramaiah, K. (2001). Cyclo-oxygenase 2: a pharmacological target for the prevention of cancer. Lancet Oncol. *2*, 544-551.
Duffy, M.J., O'Donovan, N., Brennan, D.J., Gallagher, W.M., and Ryan, B.M. (2007). Survivin: a promising tumor biomarker. Cancer Lett. *249*, 49-60.
Dvory-Sobol, H., Cohen-Noyman, E., Kazanov, D., Figer, A., Birkenfeld, S., Madar-Shapiro, L., Benamouzig, R., and Arber, N. (2006). Celecoxib leads to G2/M arrest by induction of p21 and down-regulation of cyclin B1 expression in a p53-independent manner. Eur. J. Cancer. *42*, 422-426.
Edwards, J., Mukherjee, R., Munro, A.F., Wells, A.C., Almushatat, A., and Bartlett, J.M. (2004). HER2 and COX2 expression in human prostate cancer. Eur. J. Cancer *40*, 50-55.
Erkanli, S., Bolat, F., Kayaselcuk, F., Demirhan, B., and Kuscu, E. (2007). COX-2 and survivin are overexpressed and positively correlated in endometrial carcinoma. Gynecol. Oncol. *104*, 320-325.
Feng, L., and Wang, Z. (2006). Chemopreventive effect of celecoxib in oral precancers and cancers. Laryngoscope *116*, 1842-1845.
Folkman, J. (1990). What is the evidence that tumors are angiogenesis dependent? J. Natl. Cancer Inst. *82*, 4-6.
Folkman, J., and Shing, Y. (1992). Angiogenesis. J. Biol. Chem. *267*, 10931-10934.
Gleich, L.L., Zimmerman, N., Wang, Y.O., and Gluckman, J.L. (1998). Angiogenic inhibition for the treatment of head and neck cancer. Anticancer Res. *18*, 2607-2609.
Goodin, S., and Shiff, S.J. (2004). NSAIDs for the chemoprevention of oral cancer: promise or pessimism? Commentary re J.L. Mulshine et al., randomized, double-blind, placebo-controlled, phase IIB trial of the cyclooxygenase inhibitor Ketorolac as an oral rinse in oropharyngeal leukoplakia. Clin. Cancer Res. *10*, 1565-1573.
Hao, Y., Xie, T., Korotcov, A., Zhou, Y., Pang, X., Shan, L., Ji, H., Sridhar, R., Wang, P., Califano, J., et al. (2009). Salvianolic acid B inhibits growth of head and neck squamous cell carcinoma *in vitro* and *in vivo* via cyclooxygenase-2 and apoptotic pathways. Int. J. Cancer *124*, 2200-2209.
Harris, R.E. (2007). Cyclooxygenase-2 (cox-2) and the inflammogenesis of cancer. Subcell. Biochem. *42*, 93-126.
Haupt, S., Kleinstern, J., Haupt, Y., and Rubinstein, A. (2006). Celecoxib can induce cell death independently of cyclooxygenase-2, p53, Mdm2, c-Abl and reactive oxygen species. Anti-Cancer Drugs *17*, 609-619.
Hsue, S.S., Chen, Y.K., and Lin, L.M. (2008). Expression of survivin and XIAP for DMBA-induced hamster buccal pouch squamous cell carcinogenesis is associated with p53 accumulation. Oral Oncol. *44*, 43-49.
Inoue, H., Yokoyama, C., Hara, S., Tone, Y., and Tanabe, T. (1995). Transcriptional regulation of human prostaglandin-endoperoxide synthase-2 gene by lipopolysaccharide and phorbol ester in vascular endothelial cells. Involvement of both nuclear factor for interleukin-6 expression site and cAMP response element. J. Biol. Chem. *270*, 24965-24971.
Johnstone, S., and Logan, R.M. (2006). The role of vascular endothelial growth factor (VEGF) in oral dysplasia and oral squamous cell carcinoma. Oral Oncol. *42*, 337-342.
Kennedy, S.M., O'Driscoll, L., Purcell, R., Fitz-Simons, N., McDermott, E.W., and Hill, A.D. (2003). Prognostic importance of survivin in breast cancer. Br. J. Cancer *88*, 1077-1083.
Kinugasa, Y., Hatori, M., Ito, H., Kurihara, Y., Ito, D., and Nagumo, M. (2004). Inhibition of cyclooxygenase-2 suppresses invasiveness of oral squamous cell carcinoma cell lines via down-regulation of matrix metalloproteinase-2 and CD44. Clin. Exp. Metastasis *21*, 737-745.
Kulp, S.K., Yang, Y.T., Hung, C.C., Chen, K.F., Lai, J.P., Tseng, P.H., Fowble, J.W., Ward, P.J., and Chen, C.S. (2004). 3-phosphoinositide-dependent protein kinase-1/Akt signaling represents a major cyclooxygenase-2-independent target for celecoxib in prostate cancer cells. Cancer Res. *64*, 1444-1451.
Lai, G.H., Zhang, Z., and Sirica, A.E. (2003). Celecoxib acts in a cyclooxygenase-2-independent manner and in synergy with emodin to suppress rat cholangiocarcinoma growth *in vitro* through a mechanism involving enhanced Akt inactivation and increased activation of caspases-9 and -3. Mol. Cancer Ther. *2*, 265-271.
Le Bitoux, M.A., and Stamenkovic, I. (2008). Tumor-host interactions: the role of inflammation. Histochem. Cell Biol. *130*, 1079-1090.
Lerebours, F., Vacher, S., Andrieu, C., Espie, M., Marty, M., Lidereau, R., and Bieche, I. (2008). NF-kappa B genes have a major role in inflammatory breast cancer. BMC Cancer *4*, 8-41.
Liu, C.H., Chang, S.H., Narko, K., Trifan, O.C., Wu, M.T., Smith, E., Haudenschild, C., Lane, T.F., and Hla, T. (2001). Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. J. Biol. Chem. *276*, 18563-18569.
Maeda, S., and Omata, M. (2008). Inflammation and cancer: role of nuclear factor-kappaB activation. Cancer Sci. *99*, 836-842.
Maier, T.J., Schilling, K., Schmidt, R., Geisslinger, G., and Grösch, S. (2004). Cyclooxygenase-2 (COX-2)-dependent and -independent anticarcinogenic effects of celecoxib in human colon carcinoma cells. Biochem. Pharmacol. *67*, 1469-1478.
Mann, J.R., Backlund, M.G., and DuBois, R.N. (2005). Mechanisms of Disease: inflammatory mediators and cancer prevention. Nat. Clin. Pract. Oncol. *2*, 202-210.
Mohan, S., and Epsteina, J.B. (2003). Carcinogenesis and cyclooxygenase: the potential role of COX-2 inhibition in upper aerodigestive tract cancer. Oral Oncol. *39*, 537-546.
Mori, F., Piro, F.R., Rocca, C.D., Mesiti, G., Giampaoli, S., Silvestre, G., and Lazzaro, D. (2007). Survivin and Cyclooxygenase-2 are co-expressed in human and mouse colon carcinoma and in terminally differentiated colonocytes. Histol. Histopathol. *22*, 61-77.
Ouhtit, A., Matrougui, K., Bengrine, A., Koochekpour, S., Zerfaoui, M., and Yousief, Z. (2007). Survivin is not only a death encounter but also a survival protein for invading tumor cells. Front. Biosci. *12*, 1260-1270.
Park, M.K., Hwang, S.Y., Kim, J.O., Kwack, M.H., Kim, J.C., Kim, M.K., and Sung, Y.K. (2004). NS398 inhibits the growth of Hep3B human hepatocellular carcinoma cells via caspase-independent apoptosis. Mol. Cells *17*, 45-50.
Parkin, D.M., Bray, F., Ferlay, J., and Pisani, P. (2005). Global cancer statistics 2002. CA Cancer J. Clin. *55*, 74-108.
Pyrko, P., Soriano, N., Kardosh, A., Liu, Y.T., Uddin, J., Petasis, N.I., Hofman, F.M., Chen, C.S., Chen, T.C., and Schönthal, A.H. (2006). Downregulation of survivin expression and concomitant induction of apoptosis by celecoxib and its non-cyclooxygenase-2-inhibitory analog, dimethyl-celecoxib (DMC), in tumor cells *in vitro* and *in vivo*. Mol. Cancer *18*, 5.19.
Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. (1997). Expression of cyclooxygenase-2 in human gastric carcinoma. Cancer Res. *57*, 1276-1280.
Ristimaki, A., Nieminen, O., Saukkonen, K., Hotakainen, K., Nordling, S., and Haglund, C. (2001). Expression of cyclooxygenase-2 in human transitional cell carcinoma of the urinary bladder. Am. J. Pathol. *158*, 849-853.
Sheng, H., Shao, J., Dixon, D.A., Williams, C.S., Prescott, S.M., DuBois, R.N., and Beauchamp, R.D. (2000). Transforming

growth factor-beta1 enhances Ha-ras-induced expression of cyclooxygenase-2 in intestinal epithelial cells via stabilization of mRNA. J. Biol. Chem. *275*, 6628-6635.

Shin, S., Sung, B.J., Cho, Y.S., Kim, H.J., Ha, N.C., Hwang, J.I., Chung, C.W., Jung, Y.K., and Oh, B.H. (2001). An anti-apoptotic protein human survivin is a direct inhibitor of caspase-3 and –7. Biochemistry *40*, 1117-1123.

Steinbach, G., Lynch, P.M., Phillips, R.K., Wallace, M.H., Hawk, E., Gordon, G.B., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., et al. (2000). The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. N. Engl. J. Med. *342*, 1946-1952.

Tang, D.W., Lin, S.C., Chang, K.W., Chi, C.W., Chang, C.S., and Liu, T.Y. (2003). Elevated expression of cyclooxygenase (COX)-2 in oral squamous cell carcinoma--evidence for COX-2 induction by areca quid ingredients in oral keratinocytes. J. Oral Pathol. Med. *32*, 522-529.

Ulrich, C.M., Bigler, J., and Potter, J.D. (2006). Non-steroidal anti-inflammatory drugs for cancer prevention, promise, perils, and pharmacogenetics. Nat. Rev. Cancer *6*, 130-140.

Wang, Z., Fuentes, C.F., and Shapshay, S.M. (2002). Antiangiogenic and chemopreventive activities of celecoxib in oral carcinoma cell. Laryngoscope *112*, 839-843.

Wilson, K.T., Fu, S., Ramanujam, K.S., and Meltzer, S.J. (1998). Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. Cancer Res. *58*, 2929-2934.

Woolgar, J.A., Rogers, S.N., Lowe, D., Brown, J.S., and Vaughan, E.D. (2003). Cervical lymph node metastasis in oral cancer: the importance of even microscopic extracapsular spread. Oral Oncol. *39*, 130-137.

Yamazaki, R., Kusunoki, N., Matsuzaki, T., Hashimoto, S., and Kawai, S. (2002). Selective cyclooxygenase-2 inhibitors show a differential ability to inhibit proliferation and induce apoptosis of colon adenocarcinoma cells. FEBS Lett. *531*, 278-284.

Yang, C., Zeisberg, M., Lively, J.C., Nyberg, P., Afdhal, N., and Kalluri, R. (2003). Integrin alpha1beta1 and alpha2beta1 are the key regulators of hepatocarcinoma cell invasion across the fibrotic matrix microenvironment. Cancer Res. *63*, 8312-8317.

Yang, W.T., Lewis, M.T., and Hess, K. (2010). Decreased TGF beta signaling and increased COX2 expression in high risk women with increased mammographic breast density. Breast Cancer Res. Treat. *119*, 305-314.

Zimmermann, K.C., Sarbia, M., Weber, A.A., Borchard, F., Gabbert, H.E., and Schrör, K. (1999). Cyclooxygenase-2 expression in human esophageal carcinoma. Cancer Res. *59*, 198-204.