

Differential effects of selective cyclooxygenase-2 inhibitors in inhibiting proliferation and induction of apoptosis in oral squamous cell carcinoma

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Abstract. Cyclooxygenase-2 (COX-2), an enzyme that catalyzes the synthesis of prostaglandins, is made inducible by various stimuli such as inflammation. Although COX-2 is commonly overexpressed in a variety of premalignant and malignant conditions including oral leukoplakia and squamous cell carcinoma, relatively little research has compared the effects of various COX-2 inhibitors (celecoxib, NS-398, nimesulide and meloxicam). Therefore, we investigated the effects of four different selective COX-2 inhibitors on the growth of KB cells, derived from oral squamous cell carcinoma (OSCC) and its mechanisms. Celecoxib and NS-398 strongly suppressed the proliferation of KB cells at 10-100 μ M, whereas nimesulide and meloxicam are less potent proliferation inhibitors. Only celecoxib induced apoptosis of the KB cells, as detected on the basis of DNA fragmentation, caspase-3/7 activation and cleaved poly(ADP-ribose) polymerase (PARP)

fragmentation. All four COX-2 inhibitors increased COX-2 protein expression but suppressed prostaglandin (PG) E₂ production in the KB cells, suggesting that the pro-apoptotic effect of celecoxib was unrelated to the inhibition of COX-2. Mechanistically, a high level of p53 protein and a low level of multidrug-resistant protein 1 (MRP1) and breast cancer resistant protein (BCRP) mRNA in KB cells with celecoxib may explain the differential effect of these selective COX-2 inhibitors in KB cells. Taken together, celecoxib is a good therapeutic candidate for treating OSCC through the suppression of cell proliferation and the induction of apoptosis in a COX-2 independent manner.

Introduction

Elevated cyclooxygenase-2 (COX-2) expression occurs in many carcinomas, including oral squamous cell carcinoma (OSCC) and leukoplakia, where it contributes to tumor progression (1). The survival of patients with OSCC has not improved significantly over the past three decades. The main reasons for treatment failure are the development of secondary tumors and an increase in multidrug resistance-related factors that occur as a result of the chronic exposure of patients with advanced OSCC to various drugs (2). To develop rational therapeutic strategies, it is important to identify molecular targets that are linked to the pathogenesis of OSCC. Currently, COX-2, an inducible enzyme that catalyzes the synthesis of prostaglandins (PG), is being intensively evaluated as a potential molecular target for chemopreventive agents (3).

COXs are rate-limiting enzymes involved in prostanoid production and thromboxane biosynthesis. Two COX isomers exist, COX-1 and -2, which have been postulated to be target molecules for non-steroidal anti-inflammatory drugs (NSAIDs) (4). COX-1 is a constitutively-expressed house-keeping gene, generally mediating the synthesis of PGs and is required in most tissues for normal physiological functions including the production of a protective mucus by gastrointestinal mucosa and platelet aggregation. In contrast, COX-2 is not detected in many tissues. It is an inducible immediate early gene that has been postulated to be involved not only in inflammation but also in carcinogenesis where it has an

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Abbreviations: COX-2, cyclooxygenase-2; OSCC, oral squamous cell carcinoma; PG, prostaglandin; NSAID, non-steroidal anti-inflammatory drug; EGFR, epidermal growth factor receptor; PARP, poly(ADP-ribose) polymerase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; IC₅₀, inhibition concentration 50; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel; BSA, bovine serum albumin; TBS, Tris-buffered saline; MDR1, multidrug resistance 1; MRP1, multidrug resistance-associated protein 1; BCRP, breast cancer resistance protein; RT-PCR, reverse transcription-polymerase chain reaction; PPAR γ , peroxisome proliferators-activated rector γ ; 4-NQO, 4-nitroquinoline 1-oxide; ABC, ATP-binding cassette

Key words: cyclooxygenase-2 inhibitor, oral squamous cell carcinoma, celecoxib, KB cell, apoptosis, multidrug resistance

impact on cell proliferation, differentiation, apoptosis, angiogenesis, metastasis and immunological surveillance (4,5).

NSAIDs have also been shown to exert anti-proliferative and pro-apoptotic effects on a variety of cell lines, particularly colon cancer cell lines (6,7), suggesting a possible mechanism for their chemopreventive action on the colon. Although COX is the molecular target of most NSAIDs, the anti-proliferative and pro-apoptotic actions of these drugs have been reported not only to be due to COX-dependent mechanisms (8,9) but also to COX-independent mechanisms (10-12). Accordingly, the mechanism by which NSAIDs inhibit cell proliferation and induce apoptosis remains unclear.

The up-regulated expression of COX-2 in human head and neck squamous cell carcinoma and OSCC has been reported (13). KB cells are derived from OSCC and have a high expression of epidermal growth factor receptors (EGFR), which induce tumors in nude/beige mice (14). Classic NSAIDs inhibit COX-2 and -1, resulting in the common side effect of gastric mucosal damage. To reduce the gastrointestinal side effects of NSAIDs, selective COX-2 inhibitors were developed (15) and the effect of these selective inhibitors on the proliferation and apoptosis of cancer cells has been the subject of much investigation in the past 10 years (8,11,12). However, almost all of these studies have involved the comparison of a COX-2 inhibitor to non-selective NSAIDs (or no comparison at all) in major carcinomas including lung, breast and colon cancers. Thus, relatively little is known about the comparative effects of different selective COX-2 inhibitors on oral cancer cell proliferation and apoptosis. To address this issue, we investigated the effects of four major COX-2 inhibitors (celecoxib, NS-398, nimesulide and meloxicam) on the proliferation and apoptosis of KB cells.

Materials and methods

Reagents. NS-398 and COX-2 antibody were purchased from Cayman Chemical (Ann Arbor, MI, USA). Meloxicam and nimesulide and the caspase inhibitor N-acetyl-Asp-Glu-Val-Asp-aldehyde (Ac-DEVD-CHO) were purchased from Sigma (St. Louis, MO, USA) and Promega (Madison, WI, USA), respectively. Celecoxib was purchased from LKT Laboratories (Minneapolis, MN, USA). Antibodies against poly(ADP-ribose) polymerase (PARP), p21^{Waf1} and p27^{Kip1} antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-glyceradehyde-3-phosphate dehydrogenase (GAPDH) and anti-p53 were purchased from Calbiochem (San Diego, CA, USA) and Santa Cruz (Santa Cruz, CA, USA), respectively. Streptavidin-biotin peroxidase was purchased from DAKO (Cambridge, UK). NS-398, nimesulide, meloxicam and celecoxib were dissolved in dimethyl sulfoxide (Sigma) and the final concentration of the vehicle in all cultures was 0.1%.

Human KB cell culture. KB cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA) and cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 U/ml of penicillin (Invitrogen) and 100 µg/ml of streptomycin (Invitrogen). The cells were maintained in a humidified environment of 5%

CO₂/95% air at 37°C. The cells were cultured in 60 mm² tissue culture dishes and grown to 80% confluency before use.

Immunocytochemical assessment of COX-2. For immunocytochemical COX-2 analysis of the KB cells, the streptavidin-biotin-peroxidase complex method was employed. Cells were treated with 3% H₂O₂ for 30 min to eliminate the cellular intrinsic peroxidase activity and incubated with a primary antibody against COX-2 (Cayman) (1:100 dilution in phosphate-buffered saline, PBS) for 16 h at 4°C. Cells were then washed twice with PBS and treated with streptavidin-biotin-peroxidase (DAKO) before being detected with 3,3'-diaminobenzidine HCl. The expression of COX-2 was visualized by microscopy (magnification, x100).

Cell proliferation assay. To test the effect of selective COX-2 inhibitors on cell proliferation, cells were plated into flat-bottomed 24-well plates and the density (1-2x10⁴ cells/well in 1 ml medium) was determined on the basis of the growth characteristics. After overnight incubation, triplicate wells were treated with varying concentrations of drugs for 24, 48 and 72 h. The relative percentage of metabolically-active cells to untreated controls was then determined on the basis of the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazine. The ability of cells to form formazine, by active mitochondrial respiration, was detected using a 96-well format plate reader and the absorbance was measured at a wavelength of 550 nm (A_{550 nm}). The percentage of metabolically-active cells was compared to the percentage of control cells growing in the absence of drugs in the same culture plate. The percentage of inhibition was calculated using the formula: % inhibition = (1 - OD_{test}/OD_{control}) x 100. IC₅₀s (inhibition concentration 50) were calculated by plotting the log of the percentage of inhibition values versus the drug concentrations (16).

DNA fragmentation assay. The KB cells (1x10⁴/well) were treated with the test drugs during culture for 24 h in 96-well plates containing a medium with 1% (v/v) FBS. Cytoplasmic DNA fragments, which are an indicator of apoptosis, were measured with a DNA cell death detection ELISA^{PLUS} kit (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions.

Assay of caspase-3/7 activity. The KB cells (1x10⁴/well) were treated with the test drugs in 96-well plates containing a medium with 1% (v/v) FBS for 6 h. The level of caspase-3/7 activity, in the cells, was then measured by an Apo-ONETM homogeneous caspase-3/7 assay (Promega) according to the manufacturer's instructions.

Assay of PGE₂ production. The KB cells (5x10⁵/well) were pretreated with the test drugs in 24-well plates containing a medium with 1% (v/v) FBS for 1 h. The media was then removed and the cells were washed with PBS buffer. Arachidonic acid (10 µM, Sigma) was added to the serum-free media and the cells were incubated for another hour. The PGE₂ level in the culture medium was then measured using a commercial ELISA kit (Cayman) according to the manufacturer's instructions.

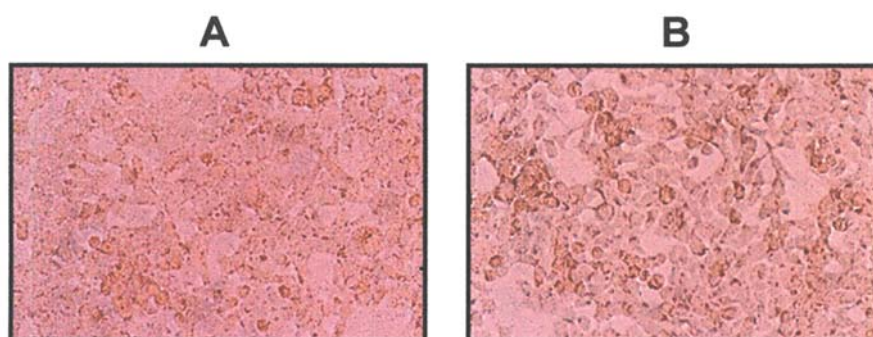


Figure 1. Cyclooxygenase-2 (COX-2) expression in KB cells. (A) Vehicle-treated KB cells (control, x100). (B) COX-2 antibody-treated KB cells (x100).

Immunoblot analysis. For immunoblot analysis, the cells were lysed in buffer consisting of 50 mM HEPES (pH 7.2), 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM $MnCl_2$, 10 μ g/ml leupeptin and 2 mM phenylmethylsulfonyl fluoride. Protein concentrations were determined using the BCA protein assay (Sigma). GAPDH (Calbiochem) was used as an internal standard for the normalization of protein loading of the immunoblots.

Protein samples (20–40 μ g protein/lane) from cells were resolved by electrophoresis on a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) and transferred to a nitrocellulose membrane (Pall Corporation, Ann Arbor, MI, USA). For immunodetection, blots were incubated overnight with the appropriate diluted primary antibody in 5% bovine serum albumin (BSA), Tris-buffered saline (TBS) and 0.1% Tween-20 at 4°C with gentle shaking. Immunoblots were then incubated with secondary antibody conjugated to horseradish peroxidase (diluted 1:10,000). The protein expression was detected by enhanced chemiluminescence (Amersham Life Science, Piscataway, NJ, USA) on Kodak X-OMAT film (Sigma). Exposed film was scanned and the bands were quantified using Kodak 1D image analysis (Eastman Kodak Company, Rochester, NY, USA) equipped with an analysis software program.

MRP1, MDR1 and BCRP expression. Total cellular RNA was isolated from KB cells using a Trizol reagent (Invitrogen) and purified with an RNeasy mini kit (Qiagen, Santa Clarita, CA, USA) according to the manufacturer's instructions. Single-stranded oligo(dT)-primed cDNA was generated from 2 μ g of total RNA in a 25 ml reaction mixture using Moloney murine leukemia virus reverse transcriptase (Promega). Primers used for the analysis of human multidrug resistance 1 (MDR1), multidrug resistance-associated protein 1 (MRP1) and breast cancer resistance protein (BCRP) were: MRP1 (sense primer 5'-CGTGTACTCCAACGCTGAC-3' and antisense primer 5'-CTGGACGCCTGACGCCC-3', 325 bp), MDR1 (sense primer 5'-GGAAGCCAATGCCTATGAC TTTA-3' and antisense primer 5'-GAACCACTGCTTCGCT TTCTG-3', 193 bp) and BCRP (sense primer 5'-TGGCTGTC ATGGCTTCAGTA-3' and antisense primer 5'-GCCACGT GATTCTTCCACAA-3', 205 bp) and GAPDH was used as an internal standard (sense primer 5'-GTCAACGGATTT GGTCTGATT-3' and antisense primer 5'-AGTCTTCTGGG

TGGCAGTGAT-3'). The amplification reaction was carried out with 2 μ l of cDNA product for 27 cycles and each cycle consisted of 94°C for 45 sec, 57°C for 50 sec and 72°C for 50 sec, followed by a final 1-min elongation at 72°C. The final polymerase chain reaction (PCR) products were electrophoresed on a 1% agarose gel.

Statistical analysis. Statistically significant differences ($p < 0.05$) between the control and each treated group were determined by a two tailed unpaired Student's t-test.

Results

Immunocytochemical assessment of COX-2. To confirm the expression of COX-2 in the KB cells, immunocytochemical analysis was carried out using the streptavidin-biotin-peroxidase complex method. COX-2 staining was not detected in vehicle-treated control KB cells (Fig. 1A) but was strongly positive in KB cells incubated with primary antibody against COX-2 (Fig. 1B). Immunoblot analysis for COX-2 also showed a strong COX-2 protein expression in KB cells (data not shown).

The effect of COX-2 inhibitors on cell proliferation. After confirming the expression of COX-2 in the KB cells, we examined the effect of four selective COX-2 inhibitors on the proliferation of KB cells by measuring the mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide to formazine (Fig. 2). The KB cells were incubated with each of the inhibitors for 24, 48 and 72 h at various concentration ranges (0–100 μ M for celecoxib and NS-398, and 0–1000 μ M for nimesulide and meloxicam, respectively). The KB cell proliferation was strongly inhibited by the four selective COX-2 inhibitors studied, in a dose- and time-dependent manner (Fig. 2A). The inhibition of KB cell proliferation was most potent following incubation with the inhibitor for 72 h and potency decreased progressively at 48 and 24 h treatment at time points (Fig. 2A). The potency for the inhibition of cell proliferation at 100 μ M for 72 h was 93.4, 55.3, 43.3 and 21.1% inhibition for celecoxib, NS-398, nimesulide and meloxicam (Fig. 2B), respectively. These results suggest that celecoxib is the strongest suppressor of KB cell proliferation. The IC_{50} values for the 72-h treatment were 34.6, 64.7, 136 and 386 μ M for celecoxib, NS-398, nimesulide and meloxicam, respectively. Again, our results

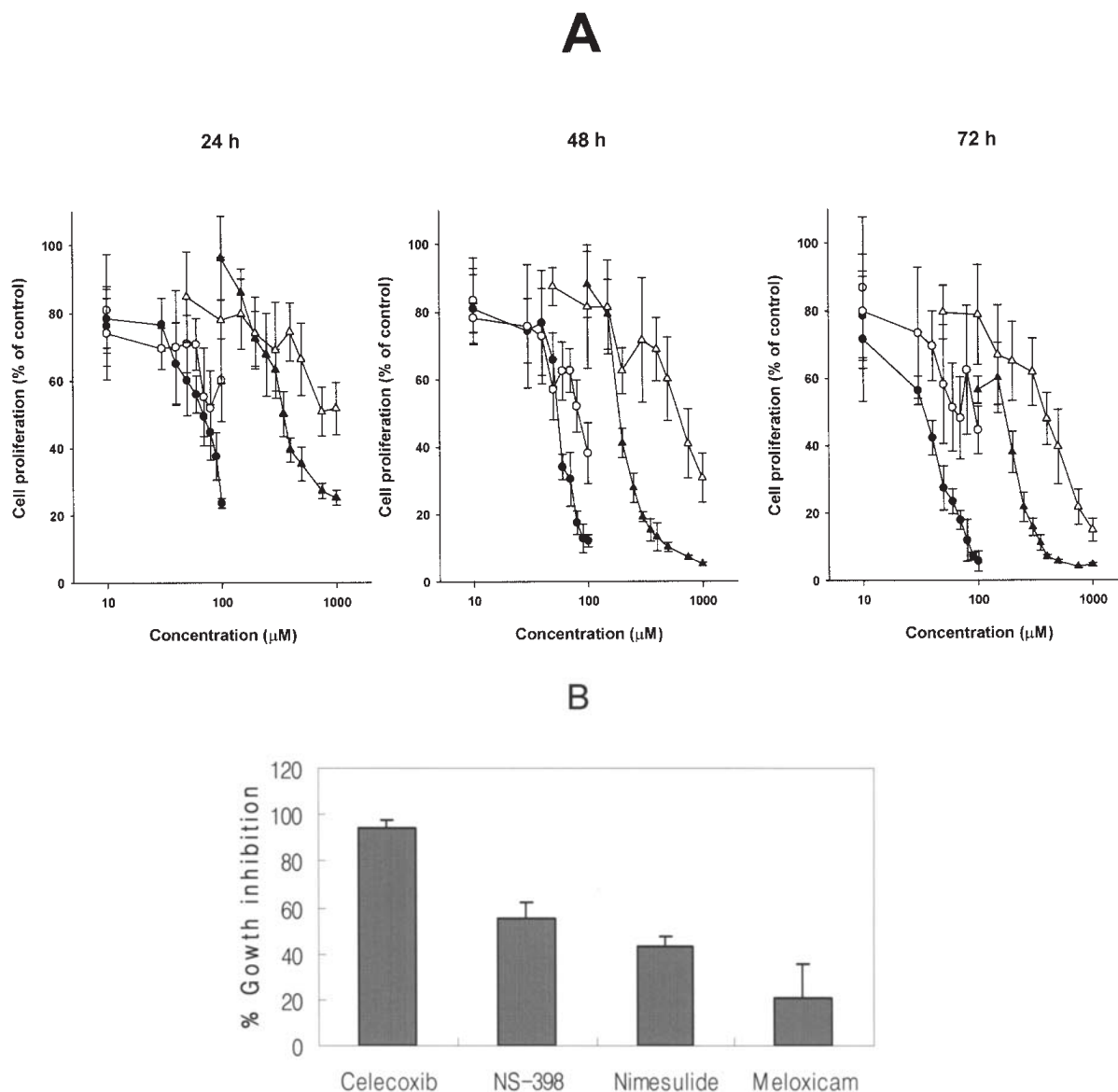


Figure 2. Effects of various COX-2 inhibitors on the proliferation of KB cells. (A) The cells were treated with celecoxib (●), NS-398 (○), nimesulide (▲) and meloxicam (△) for 24, 48 and 72 h. Cell proliferation was estimated by mitochondrial conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazine and is presented as a percentage of the control value. (B) KB cells were treated with celecoxib, NS-398, nimesulide and meloxicam at 100 μ M for 72 h. Cell proliferation was estimated as in (A) and is presented as % growth inhibition as described in Materials and methods. Data are mean \pm SD of triplicate cultures. The representative results of three independent experiments are shown.

indicate that of the four selective COX-2 inhibitors studied, KB cells were most sensitive to celecoxib.

The effect of COX-2 inhibitors on apoptosis. To determine whether the suppression of KB cell proliferation by COX-2 inhibitors is due to the induction of apoptosis, the appearance of DNA fragmentation (a hallmark of apoptosis) was quantitatively analyzed by ELISA after incubation with each inhibitor. This method specifically detects cytoplasmic histone-associated DNA fragments, mononucleosomes and oligonucleosomes. As shown in Fig. 3A, DNA fragmentation was detectable after a 4-h incubation period with 50 μ M of celecoxib. After 24 h, DNA fragmentation increased. Following exposure to the 100 μ M celecoxib, the DNA fragments were released into the culture medium, indicating

that the cells had undergone a secondary necrosis, which usually occurs after apoptosis (Fig. 3B). In contrast, NS-398, nimesulide or meloxicam, which also inhibited cell proliferation, did not influence DNA fragmentation at concentrations of up to 200 μ M and after incubation for 4 or 24 h.

The effect of COX-2 inhibitors on caspase-3/7 activation. Caspases are responsible for many of the biochemical and morphological changes that occur during apoptosis. We therefore investigated whether selective COX-2 inhibitors induce the activation of caspase-3 and/or -7 (caspase-3/7) in KB cells. As shown in Fig. 4, the incubation of KB cells with 50 μ M celecoxib for 6 h induced the activation of caspase-3/7 and this activation was completely blocked by the incubation

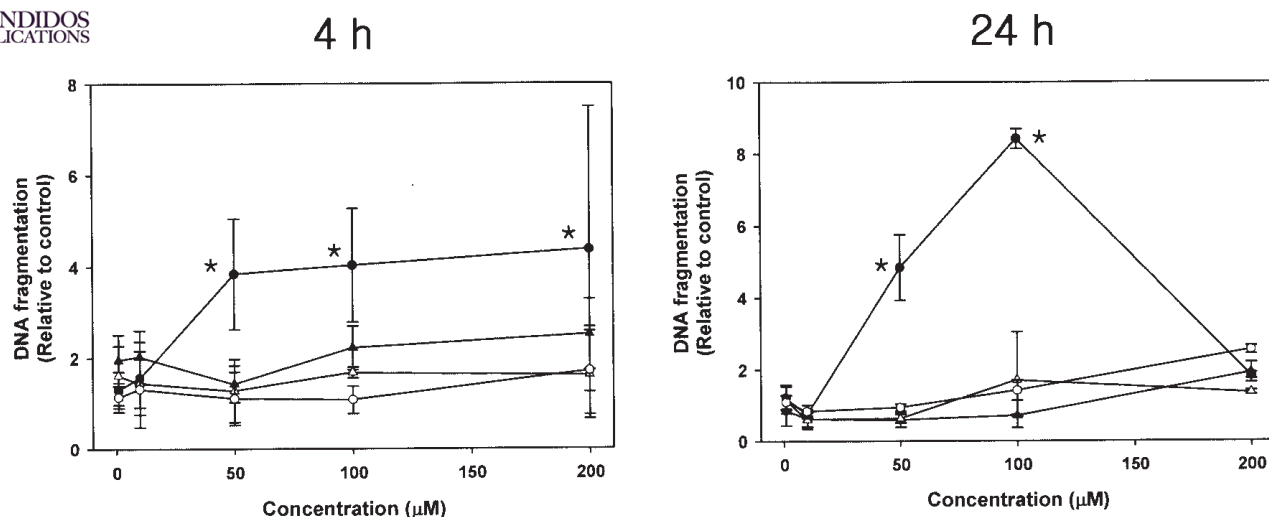


Figure 3. Effects of various COX-2 inhibitors on DNA fragmentation in KB cells. The cells were treated with celecoxib (●), NS-398 (○), nimesulide (▲) and meloxicam (△) for 4 (A) and 24 h (B). The level of DNA fragmentation in the cytoplasm was measured by ELISA. The fold induction of DNA fragmentation is shown relative to the value for the control. Data are mean \pm SD of the triplicate cultures. The representative results of three independent experiments are shown; * $p < 0.01$ compared to the control.

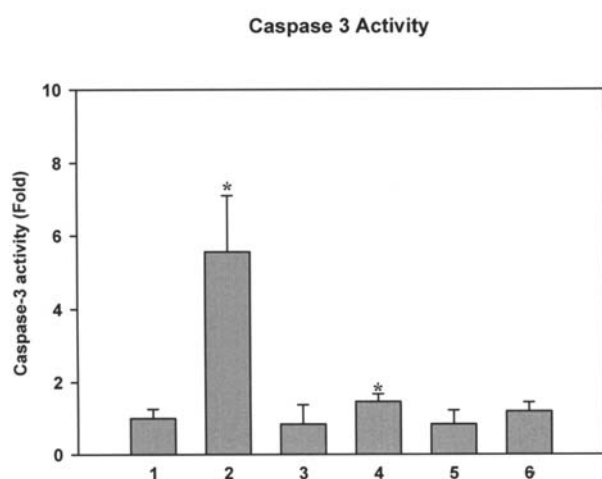


Figure 4. Effects of various COX-2 inhibitors on the caspase-3/7 activation in KB cells. The cells were cultured without COX-2 inhibitors (1) or incubated with 50 μ M celecoxib (2), 50 μ M celecoxib + 10 μ M Ac-DEVD-CHO (3), 50 μ M NS-398 (4), 200 μ M nimesulide (5) and 200 μ M meloxicam (6) for 6 h and then caspase activity was measured by the Apo-ONE™ homogeneous caspase-3/7 assay kit. The fold changes of the caspase-3/7 activity are shown relative to the value for the control. Data are the mean \pm SD of triplicate cultures. The representative results of two independent experiments are shown; * $p < 0.05$ compared to the control.

with the caspase inhibitor Ac-DEVD-CHO. In contrast, the other selective COX-2 inhibitors (50 or 200 μ M) did not alter the basal level of caspase-3/7 activity in KB cells (Fig. 4).

The effect of COX-2 inhibitors on PGE₂ production. To examine the effect of COX-2 inhibitors on the production of PGE₂ by KB cells, the cells were treated with COX-2 inhibitors for 1 h and/or stimulated with 10 μ M of arachidonic acid for another hour. As shown in Fig. 5, a concentration range of 0.5 to 50 μ M of all COX-2 inhibitors studied suppressed PGE₂ production with (Fig. 5A) or without arachidonic acid (Fig. 5B) in a concentration-dependent (celecoxib and NS-398) or

concentration-independent (nimesulide and meloxicam) manner. Selective COX-2 inhibitors with arachidonic acid suppressed PGE₂ production more potently.

The effect of COX-2 inhibitors on protein expression. To examine the effect of COX-2 inhibitors on the COX-2 protein expression, KB cells were treated with selective COX-2 inhibitors for 24 h and immunoblot analysis was performed. As shown in Fig. 6A, the protein level of COX-2 expression increased considerably by 1.51, 1.63, 2.00 and 1.79 times, compared to the control for NS-398, meloxicam, celecoxib and nimesulide, respectively. These data suggest that the effects of COX-2 inhibitors on cell proliferation or apoptosis are independent of the COX-2 protein expression.

We also analyzed the cleavage of PARP, one of the main cleavage targets of caspase-3 *in vivo* (17). As shown in Fig. 6A, the cleaved PARP fragment increased 3.3-fold in 50 μ M celecoxib-treated cells, indicating that PARP cleavage is consistent with an increased caspase-3/7 activity and DNA fragmentation in celecoxib-treated KB cells. To determine whether the effect of selective COX-2 inhibitors on KB cell proliferation is a result of cell cycle inhibition, we analyzed the cyclin-dependent kinase inhibitors, p21^{Waf1}, p27^{Kip1} and p53 using immunoblot analysis. As shown in Fig. 6B, the p21^{Waf1} and p27^{Kip1} protein expression increased significantly by 60 and 89%, respectively, in cells treated with nimesulide. Similarly, the p53 protein expression increased significantly by 98% in celecoxib-treated cells but little change was observed when the cells were treated with other selective COX-2 inhibitors.

MRP1 and BCRP expression. To investigate whether selective COX-2 inhibitors can modulate the function of multidrug-resistance related factors, we assayed the MRP1, MDR1 and BCRP mRNA expression in KB cells using reverse transcription (RT)-PCR. MRP1 and BCRP mRNA were highly expressed but the expression of MDR1 mRNA

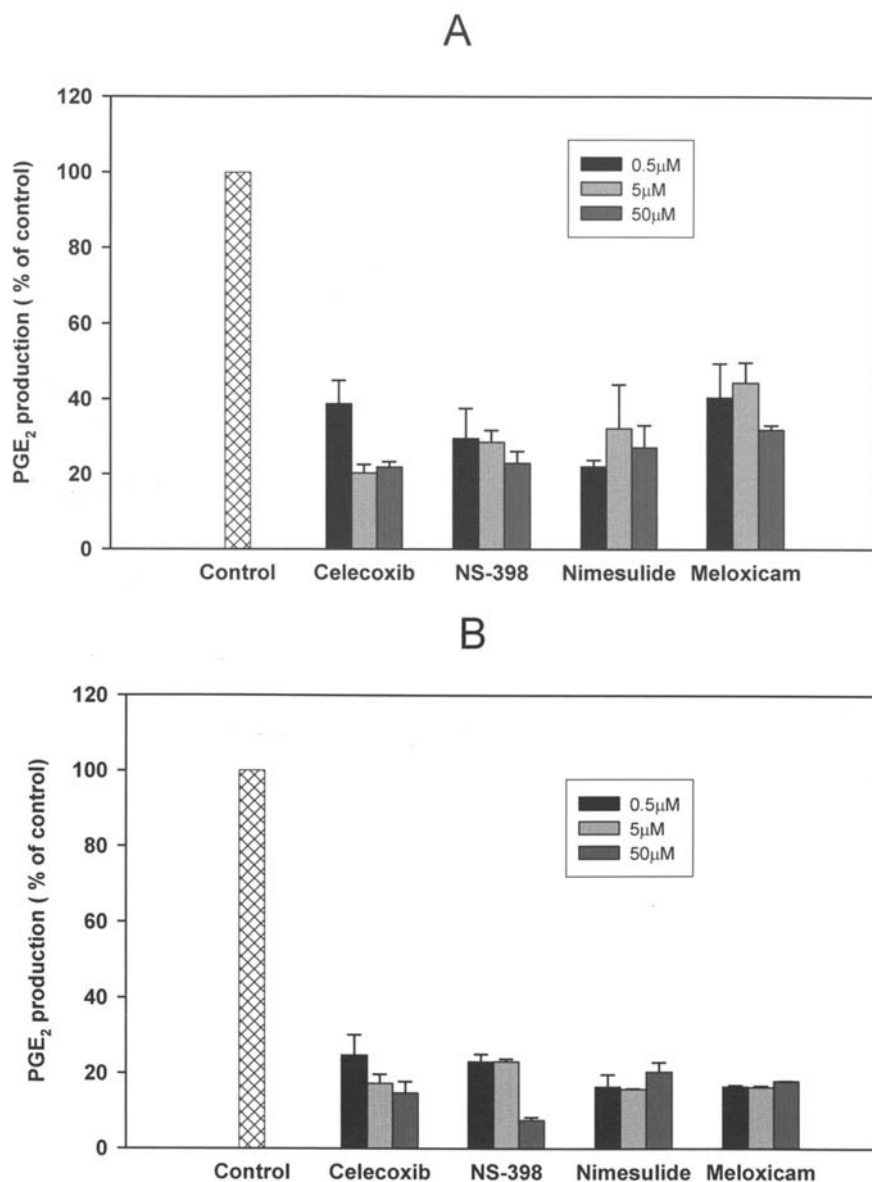


Figure 5. Effects of various COX-2 inhibitors on PGE₂ production by KB cells. (A) The cells were incubated with the selective COX-2 inhibitors for 1 h at 37°C. After incubation, the PGE₂ level was measured in the culture medium by enzyme immunoassay. Data are the mean \pm SD of duplicate cultures. The PGE₂ level in the control culture medium was 1.12 \pm 0.116 ng/ml. (B) The cells were preincubated with the selective COX-2 inhibitors for 1 h at 37°C and then 10 μ M arachidonic acid was added. After incubation for another hour, the PGE₂ level was measured in the culture medium by enzyme immunoassay. Data are the mean \pm SD of duplicate cultures. The PGE₂ level of the control culture medium was 2.50 \pm 0.204 ng/ml. The representative results of three independent experiments are shown.

was not detected in KB cells (Fig. 7). In our study, celecoxib down-regulated the expression of MRP1 and BCRP mRNA by 32 and 21%, respectively (Fig. 7). These data support the notion that the down-regulation of MRP1 and BCRP mRNA, by celecoxib, may decrease the efflux of drugs, increase the intracellular concentration of celecoxib and thus inhibit cell proliferation and induce apoptosis more effectively than the other selective COX-2 inhibitors we studied.

Discussion

In this study, we evaluated the effects and mechanisms of four major selective COX-2 inhibitors (celecoxib, NS-398, nimesulide and meloxicam) on the proliferation and

apoptosis of KB cells. We clearly showed that celecoxib and NS-398 can strongly suppress cell proliferation at the same concentration ranges (10–100 μ M). However, nimesulide and meloxicam are less potent KB cell proliferation inhibitors since a 10 times higher concentration range (100–1000 μ M) is required for the inhibition of proliferation. Among these four COX-2 inhibitors, only celecoxib induced the characteristic features of apoptosis, including DNA fragmentation, caspase-3/7 activation and cleaved PARP fragmentation in the KB cells. It has been reported that NS-398 also induces the apoptosis of colon (18), lung (19) and breast (20) carcinoma cells *in vitro*. In the present study, however, no such pro-apoptotic effect was observed, although NS-398 did inhibit cell proliferation. This discrepancy may be explained

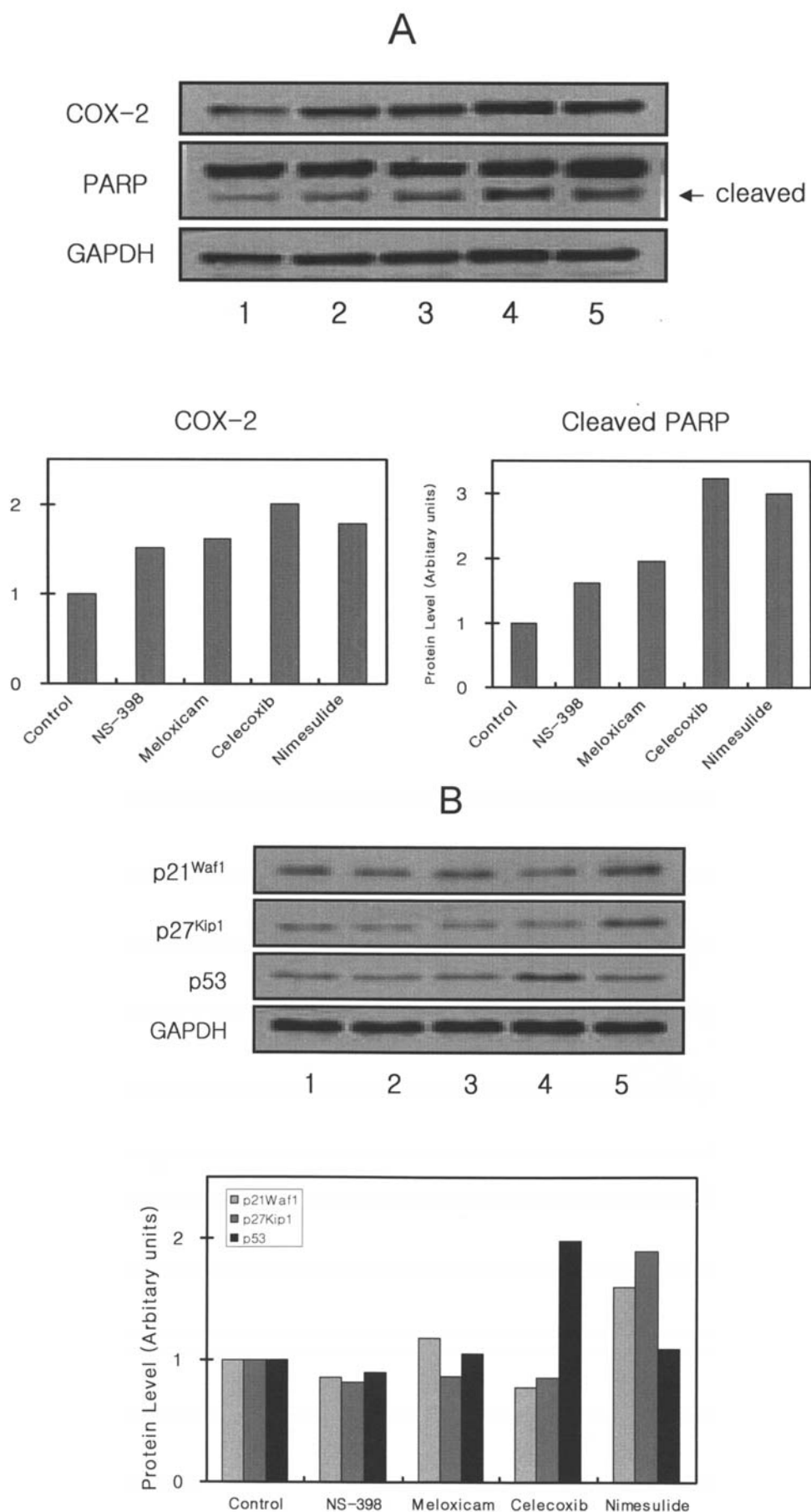


Figure 6. Effects of selective COX-2 inhibitors on protein expression in KB cells. The cells were cultured without COX-2 inhibitors (1) or incubated with 50 μ M NS-398 (2), 100 μ M meloxicam (3), 50 μ M celecoxib (4) and 100 μ M nimesulide (5) for 24 h. Protein extracts were prepared from the cells and subjected to immunoblotting, as described in Materials and methods. (A) represents the COX-2, PARP and cleaved PARP protein expression as an immunoblot (top) and graphically (bottom). (B) shows the protein expression of p21^{Waf1}, p27^{Kip1} and p53 as an immunoblot (top) and graphically (bottom).

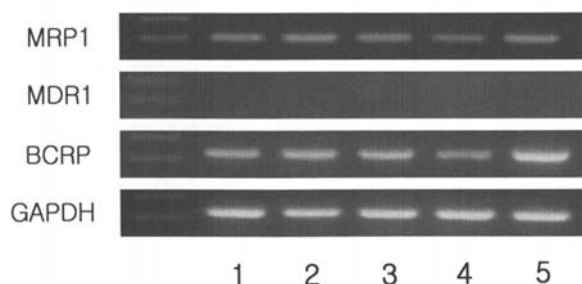


Figure 7. Effects of selective COX-2 inhibitors on the expression of MDR1, MRP1 and BCRP mRNA in KB cells. The cells were cultured without COX-2 inhibitors (1) or incubated with 50 μ M NS-398 (2), 100 μ M meloxicam (3), 50 μ M celecoxib (4) and 100 μ M nimesulide (5) for 24 h. The expression of MDR1, MRP1 and BCRP mRNA was determined in KB cells by semi-quantitative RT-PCR, with GAPDH mRNA serving as a loading control.

by differences in the carcinoma cells and the culture conditions. In the previous studies, apoptosis was determined after a relatively long culture period (more than 2 days) when compared with our study (4–24 h). In contrast, we found that celecoxib induces apoptosis after only 4 h. Taken together, these results indicate that the pro-apoptotic mechanism of celecoxib may be different from that of other selective COX-2 inhibitors.

KB cells have served as a useful model for examining the mechanisms by which NSAIDs reduce the proliferation of OSCC because the KB cells express COX-2 protein and mRNA. Although all four selective COX-2 inhibitors studied here share the ability to inhibit PGE₂ production by KB cells, these inhibitors show a differential suppression of cell proliferation and induction of apoptosis. In addition, much higher doses of these four selective COX-2 inhibitors are required to achieve inhibition of cell growth than to inhibit PGE₂ production. However, there seems to be little doubt that COX-2 contributes to tumorigenesis in OSCC, because all four COX-2 inhibitors [celecoxib (21), NS-398 (1), nimesulide (22) and meloxicam (23)] show an anti-tumor effect in animal models including those of chemically-induced carcinogenesis. These findings, combined with the present observations, suggest that the inhibition of cell proliferation and induction of apoptosis do not fully explain the mechanism by which selective COX-2 inhibitors exert an anti-tumor activity *in vivo*. It is possible that indirect mechanisms, such as an anti-angiogenic action (24), suppression of tumor invasion and down-regulation of matrix metalloproteinase-2 (1,25), may be more important than the direct effects of such agents on OSCC *in vivo*.

It is also possible that certain selective COX-2 inhibitors induce apoptosis of OSCC via targets that are not necessarily related to the COX-2 inhibitory activity. In this respect, the observation that certain NSAIDs act as a direct ligand for peroxisome proliferator-activated receptor γ (PPAR γ) is of interest (26). We found that various NSAIDs, such as indomethacin, diclofenac, oxaprozin and zaltoprofen, induce apoptosis of rheumatoid synovial fibroblasts in association with PPAR γ activation (26). Studies have also indicated that PPAR γ is expressed in OSCC, including KB cells and 4-nitroquinoline 1-oxide (4-NQO)-induced oral carcinoma

tissue. Dietary troglitazone, a selective PPAR γ ligand, inhibits 4-NQO-induced tongue carcinogenesis and suppresses an increased cell proliferation and/or COX-2 expression (27). However, we found that all four selective COX-2 inhibitors showed little change in the PPAR γ mRNA expression and did not induce a transcriptional activation of PPAR γ in a luciferase reporter assay (data not shown).

PARP is important for cells to maintain their viability. The cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis (28). In our study, cleaved PARP fragmentation was evident in celecoxib-treated cells and was followed by the induction of apoptosis in response to caspase-3 activation. These results were further confirmed by the finding that the p53 protein expression increased in celecoxib-treated KB cells, which may contribute to the inhibition of cell growth and induction of apoptosis. We also confirmed that nimesulide increased the expression of p21^{Waf1} and p27^{Kip1} proteins in KB cells and thus inhibited KB cell growth. It has been reported that celecoxib also inhibits the G₀/G₁ to S phase transition by decreasing the expression of cyclins and increasing the expression of cyclin-dependent kinase inhibitory proteins, such as p21^{Waf1} and p27^{Kip1}, in colon cancer cells (29). Recently, celecoxib was shown to induce the translation of p27^{Kip1} mRNA, which triggers the inhibition of cell growth via p27^{Kip1}-regulated cell cycle arrest in human OSCC and YD-10B cells (30).

It has not been confirmed whether celecoxib is a substrate for multidrug resistance-related factors. The multidrug resistance-related factors MRP1, BCRP and/or MDR1 are transporter proteins belonging to the ATP-binding cassette (ABC) superfamily. However, our data demonstrate that celecoxib reverses BCRP- and MRP1-related drug resistance via the down-regulation of MRP1 and BCRP mRNA. Thus, it is plausible that the efflux of celecoxib is decreased and the intracellular concentration of the drug is increased, and while not measured here, this may contribute to the increase in drug sensitivity. This may therefore be another indirect mechanism by which celecoxib inhibits cell proliferation and induces apoptosis more effectively. Recent reports have shown a multi-targeted inhibition of drug-resistant multiple myeloma cell lines by an analog of celecoxib known as dimethyl-celecoxib (31) and have also shown an inhibitory effect of celecoxib combined with or without octreotide on the growth of a multidrug resistant human gastric cancer cell line SGC7901/ADR (32).

In summary, celecoxib strongly suppresses the proliferation of KB cells and induces their apoptosis, as detected by DNA fragmentation, caspase-3/7 activation and cleaved PARP fragmentation in a COX-2-independent manner. These data also confirm that celecoxib-treated KB cells express high levels of the p53 protein and low levels of MRP1 and BCRP mRNA. Accordingly, celecoxib is a good therapeutic candidate for treating human OSCC in a COX-2 independent manner.

Acknowledgements

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