Antitumor effects of inhibitors of nitric oxide synthase or cyclooxygenase-2 on human KB carcinoma cells overexpressing COX-2

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Abstract. Inducible nitric oxide synthase (iNOS) and cyclooxygenase (COX)-2 are major inflammatory mediators. Nitric oxide (NO) produced by iNOS has been shown to have an important role in carcinogenesis. Recent studies have suggested that COX-2 expression also contributes to carcinogenesis, as well as tumor growth, invasion, and metastasis. COX-2 inhibitors such as celecoxib are widely recognized to have antitumor activity, but can cause adverse effects. We investigated possible relations between COX-2 and NO with the use of a human epidermoid carcinoma cell line, designated KB, in which overexpression of COX-2 protein was induced by gene transfer. We also assessed the possibility of using NOS inhibitor as an antitumor drug. We isolated a COX-2 transfected clone (KB/COX-2) and used a neomycin-transfected clone (KB/neo) as control. N^G-nitro-L-arginine-methyl ester (L-NAME) was used as a NOS inhibitor, dihydrochloride (1400W) as an iNOS inhibitor, and celecoxib as a selective COX-2 inhibitor. All agents inhibited the cell growth of both clones to similar extents in a dose-dependent manner. Prostaglandin E_2 (PGE_2) production and COX-2 expression in KB/COX-2 were inhibited not only by celecoxib, but also by L-NAME and 1400W. The decreases in PGE_2 production and COX-2 expression were most prominent with celecoxib and L-NAME. *In vivo*, L-NAME and celecoxib significantly inhibited the proliferation of KB/COX-2-xenografted tumors. Tumor weight was reduced by L-NAME (60.6% decrease), 1400W (38.0% decrease), and celecoxib (74.5% decrease) as compared with the control after 21 days of treatment. Immunohistochemically, xenografted tumors expressed COX-2, iNOS, and eNOS. Such expression was suppressed by treatment with L-NAME and celecoxib. These results suggest that L-NAME and celecoxib significantly inhibit the proliferation of murine squamous cell carcinoma *in vivo*. L-NAME as well as celecoxib might thus be useful for the design and development of new antitumor drugs.

Introduction

The biosynthesis and release of nitric oxide (NO) and prostaglandins (PGs) share a number of similarities. Two major forms of nitric oxide synthase (NOS) and cyclooxygenase (COX) have been identified to date. NO is a multifunctional gaseous molecule synthesized from L-arginine by NOS. There are three isoforms of NOS: neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible isoform of NOS (iNOS). nNOS and eNOS are constitutively expressed and are also referred to as constitutive NOS (cNOS). In contrast, iNOS is transcriptionally regulated and induced by inflammatory cytokines, endotoxins, hypoxia, and oxidative stress (1,2). iNOS produces high, sustained concentrations of NO, whereas the other two isoforms produce low, transient concentrations of NO (3).

Previous studies have shown positive correlations between iNOS and poor outcomes in patients with breast cancer and melanoma (4,5). These observations suggest that NO generated by iNOS has multiple physiologic and pathologic effects. It has been reported that eNOS can modulate cancer-related events, such as angiogenesis, apoptosis, the cell cycle, tumor invasion, and metastasis (6). To our knowledge, only one clinical study has examined eNOS expression in head and neck cancer (7). That study assessed the immunohistochemical expression of eNOS in patients with hyperplasia, dysplasia, and invasive cancer and reported that eNOS expression was significantly higher in cancerous and precancerous lesions than in normal oral mucosa (7).

Cyclooxygenase (COX) catalyzes the synthesis of prostaglandins (PGs) from arachidonic acid. Two isoforms of the COX enzyme exist, COX-1 and COX-2. COX-1 is expressed constitutively in many organs, including the alimentary canal, whereas COX-2 is induced by stimuli such as cytokines and growth factors and is involved in various biologic responses

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(8-11). We previously demonstrated that overexpression of COX-2 increases cell migration *in vitro* and augments tumorigenicity and local tumor invasion *in vivo* via up-regulation of MMP and Rho family small GTPases and down-regulation of TIMP activities in a human KB carcinoma cell line (12).

Inflammatory processes are mediated by multiple molecular mechanisms. Two of the most prominent are the production of NO by NOS (iNOS and eNOS) and the formation of PGs by COX-2. Given the correlation between the NOS and COX pathways, we compared the antitumor effect of NOS inhibitor with that of COX-2 inhibitor against a human KB carcinoma cell line in which malignancy was increased by gene transfer of COX-2 cDNA.

Materials and methods

Cell line and cell culture. Human KB carcinoma cell line (13) derived from epidermoid carcinoma of the floor of the mouth was used in this study. KB cells were grown in Dulbecco's modified Eagle's MEM (DMEM) (Nissui Pharmaceutical Co., Tokyo, Japan) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT, USA) and 4 mM L-glutamine as growth medium at 37˚C in a 5% CO_2 incubator. This cell line was routinely subcultured with a mixture of EDTA-trypsin. KB cells that minimally possessed COX-2 protein were transfected with full-length human COX-2 cDNA (14) (a gift of Dr R. Kulmacz, University of Texas Medical School, Houston, TX) and pcDNA3 containing a neomycin-resistant marker. The calcium phosphate method was used, as described previously (14). We isolated a clone that maximally expressed COX-2 (KB/COX-2) and also isolated a neomycin-transfected clone (KB/neo) as control. COX-2 protein expression by KB/COX-2 was ~3-4-fold higher than that of KB/neo. There was no apparent morpho-logical difference between KB/COX-2 and KB/neo.

Agents. N^G-nitro-L-arginine-methyl ester (L-NAME), a NOS inhibitor, was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Dihydrochloride (1400W), a selective iNOS inhibitor, was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). These agents were dissolved in PBS(-) before use. Celecoxib, a selective COX-2 inhibitor, was generously provided by Pfizer Inc. (New York, NY, USA). This agent was dissolved in dimethylsulfoxide (DMSO) (Dojindo Laboratories, Kumamoto, Japan) before use.

Cell growth assay. Cells were plated at $2.5x10^3$ cells/well in a 100-μl volume in 96-well plates and cultured in growth medium at 37˚C. Cell growth was assessed by 3(4,5-dimethylethiazoly 1-2-)2,5-diphonyl tetrazolium bromide (MTT) assay after 1, 3, 5, and 7 days of incubation, as described previously (15).

Effect of inhibitors on cell growth. Cells were plated at $2.5x10^3$ cells/well in a 100-μl volume in 96-well plates and cultured in growth medium at 37˚C for 24 h. Various concentrations of inhibitors were added to the wells, and the cells were further cultured for 72 h. Cell growth was assessed by MTT assay.

Prostaglandin E_2 (PGE$_2$) immunoassay. Cells were plated at $2x10^4$ cells/well in a 1-ml volume in 24-well plates and grown at 37˚C for 24 h in growth medium. The cells were then treated with inhibitors at the IC_{50} concentration for 72 h. The medium was discarded, and 1 ml of serum-free fresh medium was added to each well. The conditioned medium was collected after 15 min. PGE_2 assay was performed according to the protocol included with the prostaglandin E_2 EIA kit (Cayman Chemical), as described previously (15).

Western blot analysis. Cells and tumor samples were lysed in a lysis buffer consisting of Mg^{2+}- and Ca^{2+}-free phosphate-buffered saline (PBS), containing 20 mM Tris-HCl, pH 8.0, 1% NP40, 150 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% ß-mercaptoethanol, 0.5 mM dithiothreitol, and a mixture of proteinase inhibitors consisting of 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 5 mM benzamidine, 1 μg/ml pepstatin, 2 μg/ml antipain hydro-chloride (Boehringer, Mannheim, Germany), 50 μM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (Wako Pure Chemical Industries, Ltd., Osaka, Japan), 2 mM sodium orthovanadate (Sigma-Aldrich Co.), and 20 U/ml ulinastatin (Mochida Pharmaceutical, Tokyo, Japan). Lysates containing 15 μg protein were electrophoresed in a 10-20% gradient SDS-PAGE mini gel (Bio-Rad, Chicago, IL, USA) and blotted onto a PVDF membrane using Multiphor II (Amersham Pharmacia Biotech, Buchinghamshire, UK) for 30 min. The blotted membrane was blocked with 5% skim milk in 10 mM Tris-HCl, pH 7.2, containing 150 mM NaCl and 0.5% Tween-20 and incubated with primary antibodies (0.1-1 μg/ml) at 4˚C for 16 h as described below. The membrane was then incubated with alkaline phosphatase-conjugated secondary antibodies (0.02 μg/ml) for 4 h at room temperature as described below. The membrane was rinsed and then treated with nitroblue tetrazolium (Sigma-Aldrich Co.) and 5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich Co.) to visualize the protein bands. The primary antibodies used were rabbit polyclonal antibody against iNOS and eNOS and goat poly-clonal antibody against COX-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibodies used were anti-goat, anti-rabbit, or anti-mouse IgGs conjugated with alkaline phosphatase (Santa Cruz Biotechnology). Actin was used as internal control.

In vivo tumor xenograft study. Cells (10^6) were inoculated subcutaneously into the flanks of 5-week-old female nude mice (BALB/C nu/nu; Oriental Yeast Ltd., Osaka, Japan). Three days after KB/COX-2 xenograft implantation, when the tumor reached ~3 mm in diameter, the mice were treated with inhibitors. The treatment schedule comprised a single injection per animal, given subcutaneously every day for 21 days. The animals received L-NAME (20 mg/kg), 1400W (20 mg/kg), celecoxib (20 mg/kg), or drug vehicle (PBS[-]). The relative tumor weight (mg) was calculated by the formula $a^2xb/2$, where a is the tumor width in mm and b is the tumor length in mm, according to the method of the Battelle Columbus Laboratories (16).

Immunohistochemical study of xenografted tumors. Tissues specimens were fixed in 10% formalin, embedded in paraffin,
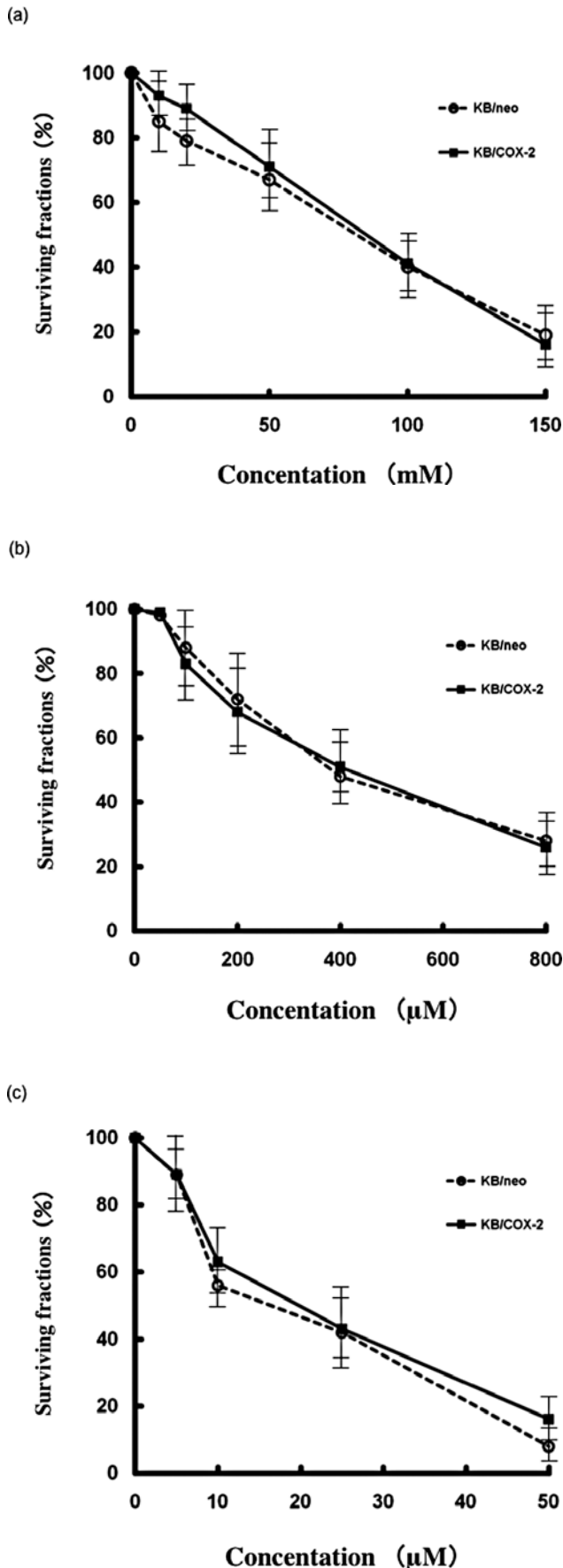
(a)

(b)

(c)

Figure 1. Cell growth inhibition by the following inhibitors: (a) L-NAME, (b) 1400W, and (c) celecoxib in KB/COX-2 and KB/neo. Cell viability was determined by MTT assay after treating cells with various concentrations of inhibitors at 37°C for 72 h. ○, KB/neo; ■, KB/COX-2. Data represent the means ± SD of 6 wells.
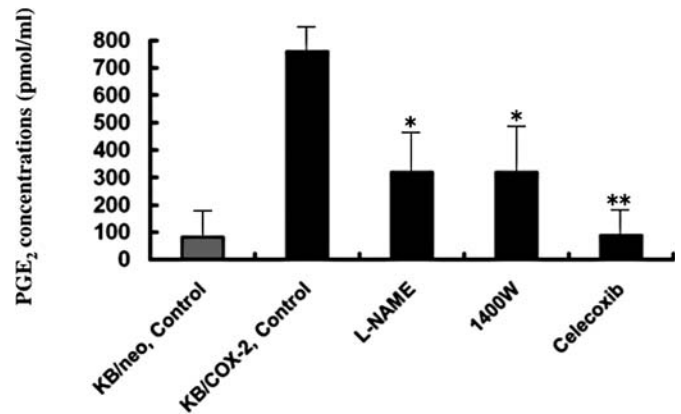

Figure 2. Effects of inhibitors on PGE_2 production in KB/COX-2. Cells grown at 37°C for 24 h were treated with inhibitors at the IC_{50} concentration for 72 h. Aliquots of culture medium were harvested, and PGE_2 levels were determined with the use of PGE_2 EIA kits. Data represent the means ± SD of 6 wells. *p<0.05, **p<0.005.

and cut into 4-μm sections according to conventional procedures. Immunohistochemical examination was performed with the use of the avidin-biotin-peroxidase complex (ABC) staining method (17). Briefly, the specimens were blocked for endogenous peroxidase activity by treatment with 0.3% H_2O_2 in methanol for 5 min. The specimens were washed and treated with 1% normal horse serum in PBS for 15 min. After washing with PBS, rabbit polyclonal antibody for human NOS3 (Santa Cruz Biotechnology), rabbit polyclonal antibody for human NOS2 (Santa Cruz Biotechnology), or rabbit poly-clonal antibody for human COX-2 (IBL, Gunma, Japan), was applied as primary antibody at 4°C overnight. After further washing with PBS, the specimens were incubated with ABC complex solution (Vectastain, Vector Laboratories, Burlingame, CA, USA) at room temperature for 15 min. After washing with PBS, biotinylated goat anti-mouse IgG (Vector Laboratories) was applied to the sections, which were then incubated for 30 min at room temperature. The specimens were treated for ~5 min with a substrate solution containing 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Ltd.) and H_2O_2. Finally, the specimens were counterstained with hematoxylin, dehydrated, and mounted with glycerol gelatin.

The iNOS, eNOS, and COX-2 labeling indexes (L.I.) were obtained by calculating the ratio of positive cells to the total number of tumor cells counted in well-labeled areas, as determined by scanning four areas at x200 magnification.

Statistical analysis. Statistical analysis was done with Student's t-test. Differences were considered significant at p<0.05.

Results

Cell growth inhibition by inhibitors. On analysis of the cell growth curves, KB/COX-2 and KB/neo showed similar growth rates. All inhibitors (L-NAME, 1400W and celecoxib) inhibited cell growth in a dose-dependent manner (Fig. 1). There was no difference between KB/COX-2 and KB/neo after treatment with any inhibitor. The IC_{50}s of L-NAME,
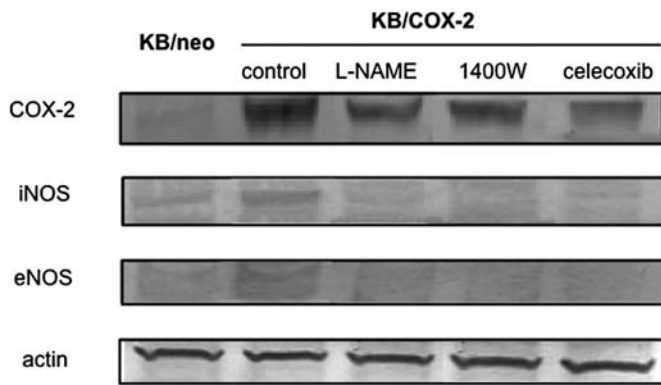
Figure 3. Expression levels of iNOS, eNOS, and COX-2 were examined by Western blot using polyclonal antibodies against these proteins. KB/COX-2 cells were grown at 37°C for 24 h and were then treated with inhibitors at the IC_{50} concentration for 72 h.

Figure 4. Effect of inhibitors on KB/COX-2 xenograft tumor volume. Cells (10^6) were inoculated subcutaneously into the flanks of five 5-week-old female mice (BALB/C nu/nu). Beginning on day 3 after KB/COX-2 challenge, the mice were given daily subcutaneous injections of L-NAME (20 mg/day), 1400W (20 mg/day), or celecoxib (20 mg/day). Tumors developing at the inoculated sites were measured with calipers once a week. The relative tumor weight was determined according to the method of Battelle Columbus Laboratories (16). ○, KB/neo; ■, KB/COX-2; □, L-NAME; ●, 1400W; ▲, celecoxib. $^*p<0.05$, $^{**}p<0.005$.

1400W, and celecoxib against KB/COX-2 were 85 mM, 410 μM, and 20 μM, respectively.

PGE₂ production. PGE_2 production by KB/COX-2 cells was ~9 times higher than that by KB/neo cells. PGE_2 production was inhibited by all inhibitors and was significantly inhibited by celecoxib (Fig. 2). These results suggested that not only COX-2 inhibitor but also NOS inhibitors reduced the amount of PGE_2 released by KB/COX-2.

Effects of inhibitors on iNOS, eNOS, and COX-2 expression in vitro. The expression of iNOS, eNOS, and COX-2 in untreated cells was compared with that in cells treated with each inhibitor by Western blot analysis. COX-2 expression in KB/COX-2 was decreased by all inhibitors. The decreases in COX-2 expression after treatment with L-NAME and celecoxib were most prominent. However, there was no difference in the expression of iNOS or eNOS between the untreated and treated cells (Fig. 3).
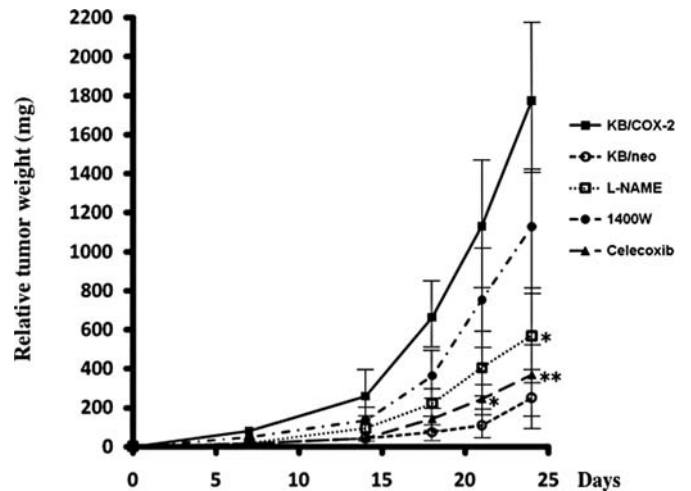
Effect of inhibitors on the growth of subcutaneously xeno-transplanted tumors of KB/COX-2 in nude mice. After subcutaneous inoculation into nude mice, the growth of KB/COX-2 tumors was significantly greater than that of KB/neo tumors. All inhibitors were well tolerated by the mice, with no weight loss or signs of toxicity. These inhibitors inhibited the proliferation of the subcutaneously inoculated tumors. In KB/COX-2 tumor-bearing mice, treatment with L-NAME for 18 and 21 days significantly inhibited tumor growth as compared with treatment with vehicle (p<0.05). Eleven days after tumor-cell challenge, celecoxib decreased tumor size as compared with vehicle-treated mice (p<0.05).
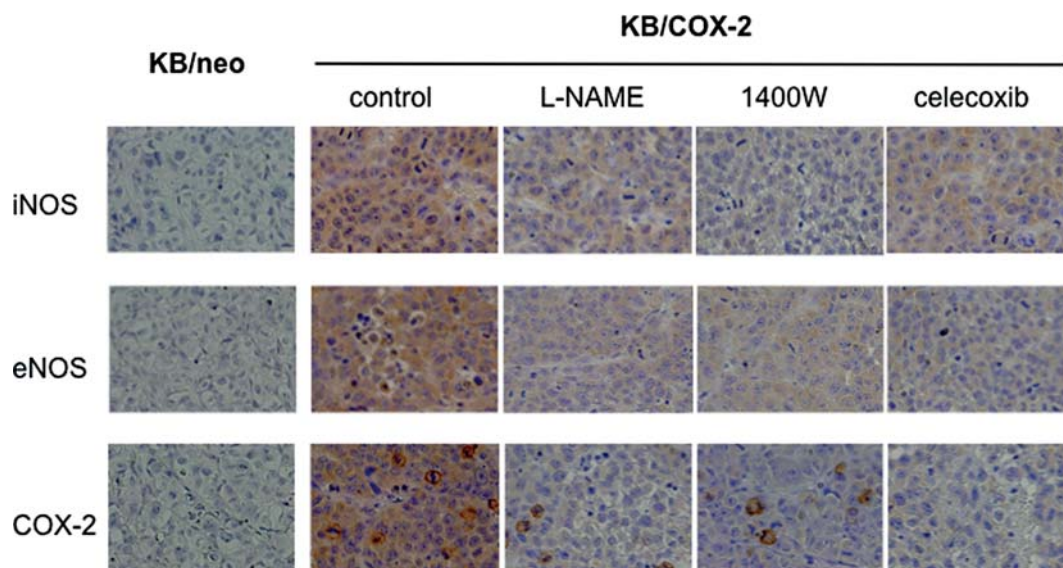

Figure 5. Immunohistochemistry of the KB/neo and KB/COX-2 tumors. KB/COX-2 tumors include tumors treated with L-NAME, 1400W, or celecoxib.
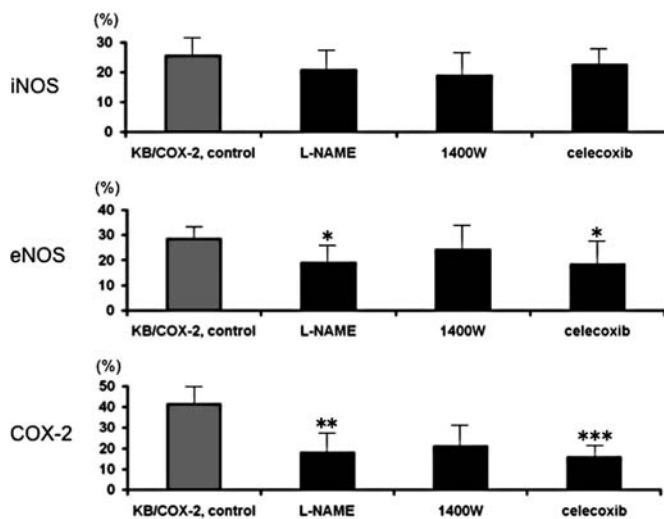
Figure 6. Labeling indexes of iNOS, eNOS, and COX-2 in KB/COX-2 tumors. *p<0.05, **p<0.005, ***p<0.001.

At the end of the observation period, the mean tumor volume per mouse was 570±404 mm³ (60.6% decrease) in L-NAME-treated mice and 369±295 mm³ (74.5% decrease) in celecoxib-treated mice, as compared with 1448±639 mm³ in vehicle-treated mice. The reduction in tumor growth did not differ significantly between L-NAME and celecoxib (Fig. 4).

Immunohistochemical evaluation. The immunoreactivity of each specimen was evaluated by light and transmission microscopy to assess the intensities of iNOS, eNOS, and COX-2 expression (Fig. 5). The iNOS L.I. was 25.5% in vehicle-treated tumors, 20.8% in L-NAME-treated tumors, 18.8% in 1400W-treated tumors, and 22.5% in celecoxib-treated tumors. There were no significant differences in L.I. for any of the inhibitors. The eNOS L.I. was 28.5% in vehicle-treated tumors and 19% in L-NAME-treated tumors. The eNOS L.I. was significantly lower in L-NAME- and celecoxib-treated tumors than in vehicle-treated tumors. The COX-2 L.I. was 41.3% in vehicle-treated tumors, 18% in L-NAME treated tumors, 21% in 1400W-treated tumors, and 15.8% in celecoxib-treated tumors. The COX-2 L.I. was significantly lower in L-NAME- or celecoxib-treated tumors than in vehicle-treated tumors (Fig. 6).

Discussion

iNOS, which produces high concentrations of NO, has been shown to mediate neoplastic transformation in models of oncogene- and chemically-induced tumorigenesis, although conflicting results have been obtained (2). The effects of iNOS expression on the biologic characteristics of cancer are unclear and difficult to define; iNOS has been reported to promote as well as inhibit carcinogenesis (18). Targeting iNOS for the prevention and treatment of cancer has been extensively studied and reviewed. Ying *et al* (6) reported that although iNOS remains a viable candidate for cancer prevention and treatment, targeting eNOS might also be a viable strategy or least deserves attention. eNOS can regulate

the expression of the proinflammatory molecules nuclear factor-κB (NF-κB) and COX-2 (19-21). Park *et al* (22) demonstrated that NO could increase COX-2 expression in many different cancer cell lines, including liver, cervical, and gastric cancer. They also reported that endogenous NO produced by NOS plays a key role in COX-2 expression and proposed that iNOS inhibitors could be used to regulate COX-2 expression in head and neck squamous cell carcinoma cells.

In our study, the expression of COX-2 in KB/COX-2 was decreased by NOS inhibitor treatment. The decrease was more prominent with L-NAME than with 1400W. Our results showed that inhibition of both iNOS and eNOS was associated with a larger decrease in COX-2 expression than that induced by iNOS inhibition alone in carcinoma cells.

Many *in vivo* and *in vitro* studies have provided evidence that COX-2 inhibitors are useful for cancer therapy, prompting various studies to investigate the structural basis underlying such activity (23). NOS inhibitors have been less extensively studied than COX-2 inhibitors for the treatment of cancer. We have shown that L-NAME, 1400W, and celecoxib reduced the rates of KB/COX-2 cell proliferation and tumor growth in mice. Treatment with L-NAME resulted in smaller tumors and lower expression of eNOS and COX-2 than did treatment with 1400W. Ying *et al* (6) reported that eNOS inhibits apoptosis and promotes angiogenesis as well as tumor cell proliferation, mobility, and invasiveness. In cell culture models, eNOS has an essential role in endothelial cell proliferation and is a central mediator of several endothelial growth stimulators, including vascular endothelial growth factor (VEGF) and PGE₂ (24,25). Both molecules activate the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and VEGF increases eNOS activity by enhancing eNOS phosphorylation (6).

In addition, several studies have shown that iNOS induction in tumor cells promotes angiogenesis (by up-regulating VEGF expression), which increases microvascular density and augments tumor progression (26,27). Continuous inhibition of iNOS by the selective inhibitor 1400W inhibits the growth of human colon cancers as well as murine breast cancers that endogenously express iNOS. However, 1400W failed to inhibit murine colon cancers that did not express iNOS at appreciable levels (28). In the present study, 1400W treatment was less effective than L-NAME or celecoxib treatment for inhibiting the growth of KB/COX-2 tumors, most likely because iNOS protein expression is lower than COX-2 protein expression in this tumor model. Angiogenesis is a key step in solid tumor progression. Since eNOS has an essential role in endothelial cell proliferation, L-NAME might have led to a greater decrease in tumor weight as compared with 1400W treatment 21 days after tumor cell challenge.

Overexpression of COX-2 in tumor cells affects angiogenesis by promoting the production of COX-2-derived PGE₂, which stimulates endothelial cell migration and angiogenesis by increasing the expression of VEGF and augmenting the proliferation of endothelial cells (28,29). Inhibition of COX-2 activity by celecoxib reduces these effects, thereby suppressing angiogenesis and decreasing tumor growth (30). Park *et al* (22) reported that cross-talk exists between COX and NOS in head and neck cancer cell lines. There are inter-

actions between COX and NOS pathways among host tissues, not only in tumor tissue (31-33).

We found that NOS inhibitor blocked COX-2 expression, and COX-2 inhibitor blocked NOS (iNOS and eNOS) expression *in vivo*. We also showed that iNOS and eNOS levels were lower in tumors from mice treated with celecoxib in those from mice treated with L-NAME and found that celecoxib induced greater down-regulation of COX-2 than did L-NAME. Celecoxib-treated tumors were slightly smaller than L-NAME-treated tumors. However, there was no significant difference in the reduction in tumor growth between L-NAME and celecoxib. These findings suggest that the NOS inhibitor L-NAME as well as the COX-2 inhibitor celecoxib might be useful for the design and development of new antitumor drugs.

Acknowledgements

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