Significance of COX-2 expression in human esophageal squamous cell carcinoma

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Cyclooxygenase-2 (COX-2) is well established to play an important role in the tumorigenesis of a variety of human cancers; however, the function of COX-2 in the development of esophageal squamous cell carcinoma (ESCC) remains less clear. Here, we determined, first, the pattern of COX-2 expression in normal esophageal mucosa, dysplasia, carcinoma in situ (CIS) and invasive SCC. Immunohistochemical analysis showed that, while COX-2 was weakly expressed, if at all, in normal squamous epithelium, strong COX-2 expression was detected as early as the stage of dysplasia and frequently in 20 of 26 (77%) CIS and 86 of 111 (77%) invasive SCC. Upregulation of COX-2 in ESCC was found to be significantly associated with tumor progression (R = 0.493, P < 0.01). Further, treatment of human ESCC cell lines (KYSE450 and KYSE510) with NS-398, a COX-2 specific chemical inhibitor, suppressed the production of prostaglandin E2 (PGE2) and induced cell growth inhibition, cell cycle arrest at the G1–S checkpoint, and the expression of cyclin-dependent kinase inhibitors p21^{waf1/cip1} and p27^{kip1}. Finally, knockdown expression of COX-2 in KYSE450 cells by a specific COX-2 siRNA dramatically inhibited PGE2 production, cell growth and, more importantly, colony formation and tumorogenesis in nude mice. Together, this study suggested that COX-2 may be involved in an early stage of squamous cell carcinogenesis of the esophagus and has a non-redundant role in the regulation of cellular proliferation and tumorogenesis of esophageal epithelial cells.

Introduction

Cyclooxygenases (COX-1 and COX-2) are the rate-limiting enzymes involved in the biosynthesis of prostaglandins (PGs). While COX-1 is constitutively expressed in most tissues, COX-2 is inducible by trauma, tumor promoters, growth factors and inflammatory cytokines (1,2). Several lines of evidence have demonstrated that COX-2 plays an important role in tumor development and progression. First, COX-2 has been reported to be upregulated in a variety of malignant tumors including colon, gastric, pancreatic and breast cancers (3–6), and high-level expression of COX-2 in tumor tissue is related to poor prognosis in several tumor types including lung cancer, breast cancer and gliomas (7–9). Further, studies in carcinogen-induced tumors and genetically modified animals have shown that non-steroidal anti-inflammatory drugs (NSAIDs) and COX-2 selective inhibitors have profound suppressive effects on tumor development (10,11). Finally, the overexpression of COX-2 is sufficient to cause tumorigenesis in animal models, and the deletion of the COX-2 gene suppresses tumor progression in mice predisposed to intestinal neoplasia (12). These findings thus provide compelling evidence that COX-2 is an obligatory player in human cancers.

Epidemiological studies indicated that the regular use of aspirin can reduce the risk of esophageal cancer up to 90% (13,14). Considering that the best-known biochemical function of NSAIDs is to inhibit the COX activity, these studies imply that COX-2 is involved in the development of esophageal cancer. Indeed, Shamma et al. (15) found that expression of COX-2 was upregulated in a subset of dysplastic and neoplastic esophageal epithelial cells. Similarly, we and others showed the expression of COX-2 in esophageal squamous cell carcinoma (ESCC) tissue samples, although the reported COX-2-expressing stages during the ESCC development were not consistent (15–19). Therefore, analysis of the pattern of COX-2 expression in different stages of neoplasia will help us in understanding the role of COX-2 during esophageal carcinogenesis.

The role of COX-2 in malignant tumors has been investigated in many different experimental systems. Previous studies revealed that the functions of the COX-2 gene are complex and may involve different mechanisms depending on the cell types and the conditions studied. COX-2 can contribute to inhibition of apoptosis, inhibition of proliferation, increased angiogenesis, increased adhesion, increased invasion and modulation of inflammation (20). However, the molecular events mediated by COX-2 in ESCC remain largely unknown.

In the present study, we analyzed the precise pattern of COX-2 expression in normal esophageal mucosa, dysplasia, carcinoma in situ (CIS), and invasive SCC. We then studied the effects of NS-398 (N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide), a chemical inhibitor of COX-2, and a specific COX-2 siRNA in ESCC cells. These results showed that COX-2 expression could be upregulated at an early stage of squamous cell carcinogenesis of the esophagus and that inhibition of COX-2 functions was effective on the suppression of the cancer cell proliferation and tumorigenesis in nude mice.
Materials and methods

Tissue specimens
A total of 250 esophageal mucosa biopsies and ESCC cases were retrieved from the archival files at the Cancer Hospital of the Chinese Academy of Medical Sciences. This study was approved by the Institutional Review Board of the Cancer Institute of the Chinese Academy of Medical Sciences. None of the patients had received radiotherapy or chemotherapy before intervention.

After obtaining informed consent, patients were interviewed to obtain information on demographic characteristics, and clinical data were collected. Fresh specimens were taken immediately after surgical removal and stored at −80°C until the analysis. Adjacent grossly normal mucosa was also collected. A portion of each surgical tissue specimen was fixed in buffered 4% paraformaldehyde and embedded in paraffin for histological analysis and immunohistochemical studies.

Immunohistochemistry
For antigen retrieval, sections were heated for 10 min at 95°C using microwave. Endogenous peroxidase activity was blocked by incubation with 0.3% H2O2 solution for 30 min. The sections were then incubated at 4°C overnight with a mouse monoclonal antibody to COX-2 (Zymed Laboratories, San Francisco, CA) at a concentration of 6.7 μg/mL. Immunohistochemical staining was performed using a Histostain™-plus kit (Zymed, Burlingame, CA). No significant staining was observed when the same class of normal immunoglobulin at the same concentration was used as a negative control for immunohistochemical staining.

For evaluation of immunohistochemical staining, the expression levels were graded on a scale from negative to ++ as follows: 0, no positive staining; ±, only a few scattered positive cells; +, cluster(s) of positively stained cells, but accounting for <30% of the cells within a visual field; ++, cluster(s) of positively stained cells that accounted for >30% of the cells within a visual field.

Cell culture
The esophageal cancer cell lines KYSE series were generous gifts from the Cancer Hospital of the Chinese Academy of Medical Sciences. They were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). The cells were plated for 24 h for attachment, and then treated with 0.3% trypsin. After 0.3% trypsinization, the cells were washed twice with PBS, centrifuged, and resuspended in fresh medium containing 10% FCS and cultured in a humidified incubator at 37°C in a 5% CO2 atmosphere.

For transient transfection, the transfected cells were selected with 200 μg/mL of puromycin and 400 μg/mL of G418. Cell clones over 50 cells were considered to be stable clones and used for the following studies.

Semi-quantitative RT–PCR
COX-1 and COX-2 transcript levels were evaluated using semi-quantitative RT–PCR as described previously (14). The PCR reaction conditions, with a defined number of cycles and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, were individually optimized for each gene product studied. The primer sequences and PCR product sizes were as follows:

COX-1

- Forward: 5'-GCT GTG CTT GGT GCT TT-3' (226 bp)
- Reverse: 5'-AGG TCG TAT CTA CAC CT-3' (299 bp)

COX-2

- Forward: 5'-CAG TTC TGC CAT CAG TT-3' (233 bp)
- Reverse: 5'-AAG CTT CTC CGG TAC TCA TT-3' (803 bp)

MTT assay
To evaluate the effects of NS-398 on the proliferation of ESCC cell lines (KYSE450 and KYSE510), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out. Briefly, 1.5 × 104 cells were seeded in 96-well microplates and cultured for 24 h. Then, NS-398 was added to the wells as per the indicated concentrations. The cells were cultured for another 48 h and the medium in each well was then replaced with 200 μL of fresh medium containing 1 mg/mL MTT. After an additional incubation for 4 h the medium was discarded and 200 μL of DMSO was added to each well to dissolve formazan crystals. Then, the optical density was read with an automated microplate reader at 570 nm. Experiments were carried out in triplicate, and the results were shown as mean ± SD of three independent experiments.

Function of COX-2 in esophageal carcinoma

To examine the effects of NS-398 on the proliferation of ESCC cell lines (KYSE450 and KYSE510), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay was carried out. Briefly, 1.5 × 104 cells were seeded in 96-well microplates and cultured for 24 h. Then, NS-398 was added to the wells as per the indicated concentrations. The cells were cultured for another 48 h and the medium in each well was then replaced with 200 μL of fresh medium containing 1 mg/mL MTT. After an additional incubation for 4 h the medium was discarded and 200 μL of DMSO was added to each well to dissolve formazan crystals. Then, the optical density was read with an automated microplate reader at 570 nm. Experiments were carried out in triplicate, and the results were shown as mean ± SD of three independent experiments.

Enzyme-linked immunosorbent assay (ELISA) analysis of PGE2
To examine PGE2 production, 1 × 105 cells were seeded into each well of a 24-well plate in RPMI 1640 with 10% fetal calf serum (FCS) and cultured for 24 h. After 24 h, cells were incubated with NS-398 (1, 0, 10, and 100 μM) for 48 h. Supernatants were harvested, centrifuged at 8000 r.p.m. for 10 min and stored at −80°C. Concentrations of PGE2 in cell culture supernatants were determined using a PGE2, ELISA kit (Cayman Chemical, Ann Arbor, MI) according to the manufacturer’s instructions. The optical density was read in an automated microplate reader at 405 nm. The concentration of PGE2 was determined from the calculated standard curve. The experiment was performed in triplicate and the mean PGE2 value was taken as the result.

Cell cycle analysis by flow cytometry
The flow cytometry assay was performed by propidium iodide staining. KYSE450 and KYSE510 cells were seeded at a density of 3 × 105 in 5 ml culture medium. After 24 h, the cells were incubated with NS-398 (100 μM) for 48 h. Cells and supernatants were collected together and centrifuged at 1000 r.p.m. for 5 min. The pellets were washed twice with ice-cold phosphate-buffered saline (PBS) and fixed overnight at 4°C in 70% ethanol. After they were washed twice with PBS, cells were incubated with 5 μg/ml propidium iodide and 50 μg/ml RNase A in PBS for 1 h at room temperature. A flow cytometer (FACSCalibur, Becton Dickinson, Mountain View, CA) was used to perform flow cytometry. A total of 100 000 events were measured per sample. The experiment was performed in triplicate.

Immunofluorescence analysis
For immunofluorescence analysis, KYSE450 cells were treated with NS-398 (100 μM) for 48 h, then fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100 for 5 min. The sections were then incubated with specific mouse monoclonal antibodies against human COX-2 (22), p21mipa1/cip1 and p27kip1 (Zymed Laboratories, respectively), at 4°C overnight. Then, slides were washed in PBS and incubated with secondary antibodies. Nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Negative control sections were incubated with non-immune mouse immunoglobulin instead of the primary antibodies. Slides were examined under a fluorescence microscope. Data were captured and analyzed using Adobe Photoshop 7.0.

Examination of colony formation
Cells were digested to a single cell suspension. A total of 150 cells were plated in 60 mm culture dishes and kept for 14 days in a medium containing 25G18 to allow colony formation, and the medium was changed once every week. The plates were taken out to observe the colony formation after ~2 weeks, fixed in methyl alcohol and stained with Giemsa. Cell clones over 50 cells were counted using a grid. The colony formation efficiency was calculated as follows:

Colony formation efficiency = (Colonies formed/Cells seeded) × 100%

Three independent experiments were performed.

Tumorigenicity in nude mice
Cells transfected with pSilencer-COX-2 or pSilencer-negative control plasmid were trypsinized and single cell suspensions prepared. The cell viability was >95% as determined by trypan blue staining. Cells (4 × 105) in 0.1 ml of PBS were inoculated subcutaneously into the right flank of 4- to 5-week-old female
BALB/c nude mice (five for each group). At the end of 45 days, all mice were killed and weight was measured (All procedures for animal experimentation were approved by local animal research authorities and animal care was in accordance with institutional guidelines).

Statistical analysis
Statistical analysis was performed using the SPSS statistical software (SPSS). The correlation between COX-2 expression and progressive nature of the tumor was determined by Spearman’s rank coefficient. All tests were two-sided. Data from MTT assay, ELISA analysis of PGE2, clonogenecity assay and tumorigenecity assay were presented as mean \( \pm \) SD. The results were considered as significant if the \( P \)-value was \( < 0.05 \).

Results
Expression of COX-2 in the progression of ESCC
Immunohistochemical analysis was carried out to evaluate COX-2 expression in the four different stages of ESCC development: normal squamous epithelium, dysplasia, CIS and SCC of the esophagus. While weak COX-2 staining was detected in 21 of 101 (21%) normal squamous epithelium, strong COX-2 staining was observed in 7 of 12 (58%) dysplasia (Figure 1B and C), 20 out of 26 (77%) CIS (Figure 1D) and 86 out of 111 (77%) invasive SCC (Figure 1E–H). The upregulation of COX-2 expression in ESCC was significantly associated with the progression of carcinoma development (Table I, \( R = 0.493, P < 0.01 \)). No significant immunoreactivity of COX-2 was observed in the stroma. Tissue sections stained with normal immunoglobulin showed a clean background and no evidence of positive staining (Figure 1).

In our study, the staining of COX-2 protein was predominantly localized in the basal layer of the normal esophageal epithelium (Figure 1A). In the neoplastic epithelial cells, the immunoreactivity was predominant in the peripheral region of the tumor nest rather than in the inner region (Figure 1E). In addition to neoplastic epithelium, COX-2 was also observed to be expressed in the vascular endothelial cells (Figure 1H).

COX-2 expression in esophageal squamous carcinoma cell lines
To further investigate the role of COX-2 in esophageal squamous carcinoma cell lines, the COX-2 mRNA level in 11 human esophageal cancer cell lines was determined. RT–PCR analysis showed that all of these cell lines expressed COX-2 mRNA with KYSE450 and KYSE70 cells having the highest levels. On the other hand, the COX-1 mRNA level was

<table>
<thead>
<tr>
<th>Tissues diagnosis</th>
<th>COX-2 positive cases (%)</th>
<th>Correlation ( R ) ( (P) )</th>
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<tbody>
<tr>
<td>Normal epithelium</td>
<td>49 (49) 31 (31) 20 (20) 1 (1)</td>
<td>0.493 (0.000)</td>
</tr>
<tr>
<td>Dysplasia</td>
<td>3 (25) 2 (17) 6 (50) 1 (8)</td>
<td></td>
</tr>
<tr>
<td>CIS</td>
<td>2 (8) 4 (15) 11 (42) 9 (35)</td>
<td></td>
</tr>
<tr>
<td>Invasive carcinoma</td>
<td>12 (11) 13 (12) 40 (36) 46 (41)</td>
<td></td>
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Fig. 1. Immunohistochemical analysis examining the expression of COX-2 in different stages of ESCC development. Weak COX-2 staining in normal esophageal epithelium (A). Strong COX-2 staining in esophageal squamous dysplasia (Left arrow, dysplasia; right arrow, normal mucosa; B and C). CIS (D) and invasive SCC (E–G). (E), (F) and (G) show representative example of COX-2 protein staining in carcinoma with well, moderate and poor differentiation, respectively. Cytoplasmic staining for COX-2 was detected in the vascular endothelial cell (arrow, H). Immunoreactivity was lost when the antibody to COX-2 was replaced by non-immune serum (I). Original magnification: (A and C–I) \( \sim \times 200 \) and (B) \( \sim \times 100 \).
highest in COLO680N, TE12 and EC9706 followed by KYSE450 cells (Figure 2). KYSE450 and KYSE510 cell lines were selected for further experiments.

**Evaluation of the effects of NS-398 in KYSE450 and KYSE510 cells**

To evaluate the effects of NS-398 in esophageal carcinoma cells, PGE2 production, cell proliferation and cell cycle distribution were determined. NS-398 was used to treat KYSE450 and KYSE510 cells at the concentrations of 0.1, 1.0, 10 and 100 μM. In both cell lines, after 48 h of the treatment, PGE2 production was significantly suppressed by NS-398 compared with the control (P < 0.05) (Figure 3A). No further significant reduction in PGE2 level was seen when the dose was increased from 0.1 to 100 μM. MTT proliferation assay showed that the treatment of NS-398 at 100 μM caused significant inhibition of the cell proliferation, whereas no inhibition of cell proliferation was observed at the concentrations of 0.1, 1.0 and 10 μM (Figure 3B).

To further document the mechanisms by which NS-398 inhibited cell growth, flow cytometry was performed to determine the cell cycle progression in cells treated with 100 μM NS-398 for 48 h. As shown in Figure 3C, NS-398 induced a significant inhibition of cell cycle progression at the G1–S checkpoint, with no effect on G2–M transition. These results suggested that NS-398 mediated the inhibition of growth of esophageal carcinoma cells, at least in part, through cell cycle arrest at the G1–S checkpoint.

Since p21^waf1/cip1 and p27^kip1 play important roles in the G1–S checkpoint, Immunofluorescence analysis was performed to examine whether NS-398 treatment alters their expression. Figure 3D showed that the protein levels of p21 and p27 in KYSE450 cells were significantly elevated after treatment with 100 μM NS-398. These results suggested that p21^waf1/cip1 and p27^kip1 were involved in the NS-398-mediated G1–S arrest in human esophageal carcinoma cells.

**The inhibitory effects of siRNA-mediated knockdown expression of COX-2 on PGE2 production, cancer cell growth and tumor formation in nude mice**

To further study the role of COX-2 in the esophageal squamous carcinoma cells, we used the pSilencer system to stably express siRNA to suppress the expression of COX-2. KYSE450 cells were transfected with the expression plasmid of pSilencer-COX-2 or the pSilencer negative control. The transfected cells were selected with puromycin and cultured for ~1 month. We examined, first, the COX-2 mRNA and protein levels in the stable transfectants. As shown in Figure 4A and B, RT–PCR and immunofluorescence analyses revealed that the levels of both COX-2 mRNA and protein were reduced in the cells transfected with pSilencer-COX-2 (S1 and S2 clones) compared to those with the pSilencer control plasmid (Vector).

Since KYSE450 cells express COX-1 and COX-2, both of which are capable of producing PGE2, specific inhibition of COX-2 by siRNA could be used to investigate whether COX-2 contributed importantly to PGE2 production. As shown in Figure 4C, inhibition of COX-2 expression by siRNA resulted in a significant reduction of PGE2 production as measured by ELISA analysis. While control cells produced 3292 ± 986 pg/ml PGE2, the level of PGE2 in S1 and S2 clones was reduced to 1322 ± 433 and 1112 ± 355 pg/ml, respectively (Figure 4C).

To determine whether COX-2 siRNA could serve as a therapeutic agent against ESCC tumor formation in nude mice, we first tested the effects of siRNA-mediated knockdown expression of COX-2 on the growth of KYSE450 cells. Stable expression of COX-2 siRNA in KYSE450 cells resulted in a significant decrease in cell growth (Figure 5A) and colony formation (Figure 5B and C). Knock down of COX-2 by RNA interference (RNAi) failed to alter the cell cycle and the level of p21^waf1/cip1 and p27^kip1 (Figure 5D and E). Further, mice that received cells stably expressing COX-2 siRNA developed smaller tumors than those that received cells expressing control siRNA (Figure 5F and G).

**Discussion**

Tumorigenesis is a complex and multistage process. Determination of the expression pattern in the different stages of neoplasia is thus an important aspect to evaluate the role of the gene in the development of cancer. The rationale of using COX-2 selective inhibitors for cancer prevention or treatment is based on the observations that COX-2 is highly expressed in a majority of human tumors. To address whether COX-2 selective inhibitors could be potentially used for prevention or treatment of ESCC, the extent to which COX-2 is overexpressed and whether this is a feature of either specific subsets or stages of ESCC need to be determined. Our immunohistochemical analysis showed that, while COX-2 was weakly expressed, if at all, in normal squamous epithelium, strong COX-2 expression was detected in 7 of 12 (58%) dysplasia, the earliest stage of esophageal carcinogenesis, and detected more frequently at the late stages of 20/26 (77%) CIS and 86/111 (77%) invasive SCC. These findings suggested that COX-2 might be involved in the early stage of ESCC carcinogenesis. Our observations of high-level expression of COX-2 in the dysplastic and neoplastic cells are consistent with the studies reported by Yu et al. (17). However, Shamma et al. (15) reported a significantly higher COX-2 expression in high-grade dysplasia compared with other lesions. The difference in the existing data might be partly due to the source of the samples and the different antibodies used.

In this study, the staining of COX-2 protein was observed to be predominantly localized in the basal layer of normal epithelial cells and high-level expression of COX-2 appeared predominant in the peripheral region of the tumor nest rather than in the inner region. The expression of COX-2 protein in these proliferation compartments suggested that COX-2 may play a role in the cellular proliferation of the esophageal epithelial cells. This was substantiated by the significant inhibitory effects of NS-398 on cellular proliferation of...
Fig. 3. Effect of COX-2 specific inhibitor on PGE₂ production, cell proliferation, cell cycle distribution and the expression of cell cycle regulators. KYSE450 and KYSE510 cells were incubated with NS-398 (0.1, 1.0, 10 and 100 μM) for 48 h. (A) Inhibition of PGE₂ production by different concentrations of NS-398. The amount of PGE₂ released in culture medium was quantified by EIA. Values are expressed as mean ± SD of three experiments (n = 3). Significant difference from control group (P < 0.05). (B) Inhibition of cell proliferation of KYSE450 and KYSE510 cells by NS-398 (100 μM). Cells were measured for cell proliferation at OD570 as illustrated in the Materials and methods section. Values are expressed as mean ± SD of three experiments (n = 3). Significant difference from control group (P < 0.05). (C) NS-398 (100 μM) induced an increase in G1 phase and a decrease in S phase cells compared with DMSO-treated control cells. Cells were labeled with PI and analyzed by DNA flow cytometry. (D) Immunofluorescence analysis showed that both p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup> were upregulated by NS-398. KYSE450 cells grown on cover slips treated with NS-398 (100 μM) or DMSO for 48 h, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. p21<sup>waf1/cip1</sup> and p27<sup>kip1</sup> are shown in green and nuclei were counterstained with DAPI.
ESCC cells (23). NS-398 is a sulphonamide derivative that specifically inhibits COX-2 activity, with an IC$_{50}$ of 30 nM. This agent does not affect COX-1 activity at a concentration of 100 μM and has a minimal effect on the inhibition of COX-1 prostanoid production even at a dose exceeding 200 mg/kg (24,25). NS-398 at the concentrations of ~0.1-10 μM was effective on suppression of PGE$_2$ secretion while not inhibiting cell growth. The observations that the effective inhibition of cellular proliferation required at least 10-fold higher concentration (100 μM) of NS-398 than the inhibition of PGE$_2$ production did suggest that a non-COX-target(s) might be involved in NS-398 actions.

Our cell cycle analysis showed that NS-398 induced G1/S arrest in ESCC, the effects similar to those observed in pancreatic tumor cell lines and ovarian carcinoma cells (26,27). It has been shown that treatment with NSAIDs results in the upregulation of cyclin-dependent kinase inhibitors (CDKIs) that lead to accumulation of cells in G$_0$/G$_1$ (28,29). In this study, p21$^{\text{waf1/cip1}}$ and p27$^{\text{kip1}}$ were significantly elevated after treatment with 100 μM NS-398. These results suggested that p21$^{\text{waf1/cip1}}$ and p27$^{\text{kip1}}$ might contribute to COX inhibitor-mediated G1-S arrest in human esophageal carcinoma cells.

Inhibition of apoptosis is regarded as one of the mechanisms by which COX-2 promotes tumorigenesis (30). Previous studies showed an induction of apoptosis by COX-inhibitors in a variety of human cancer cell lines in vitro (23,31,32). For example, it has been shown that COX-inhibitors induce apoptosis without affecting cell cycle distribution in colon cancer cells (33). In the present study, treatment of NS-398 did not induce marked apoptosis. The effects of COX-inhibitors on different types of tumor cells are variable and may depend on the cell type and the inhibitors.

In addition to neoplastic esophageal epithelium, expression of COX-2 was also detected in the angiogenic microvasculature present within the tumors and the preexisting vasculature adjacent to tumor tissues. The expression of COX-2 in the angiogenic blood vessels of esophageal cancer suggested that COX-2 may promote angiogenesis and lack of COX-2 may prevent new blood vessel formation in esophageal epithelium (34). The function of COX-2 in promoting angiogenesis during esophageal carcinogenesis needs to be further determined.

To further dissociate the effects of NSAIDs from COX-2, we have used a DNA vector-based RNAi technique to specifically knock down COX-2 expression in esophageal carcinoma cells. This technology can be adapted to analyze gene function over a long period of time through stable inhibition (35-37). As shown here, the expression of siRNA by this vector resulted in the efficient and specific downregulation of COX-2 mRNA and protein and, as a result, in a significant reduction of PGE$_2$ production. Significantly, siRNA-mediated knockdown expression of COX-2 inhibited cellular proliferation, colony formation and tumor formation in nude mice. These studies
suggested a non-redundant role of COX-2 in the regulation of cellular proliferation and tumorigenesis of esophageal epithelial cells and pointed to the potential to use chemical inhibitors and specific siRNAs of COX-2 for prevention or treatment of ESCC. The fact that treatment of NS-398 but not knock down of COX-2 by siRNA can alter the level of p21\textsuperscript{waf1/cip1} and p27\textsuperscript{kip1} in S1 and S2 clones or Vector cells. There were no obvious changes after transfection. p21\textsuperscript{waf1/cip1} and p27\textsuperscript{kip1} are shown in green and nuclei were counterstained with DAPI. (F and G) Knock down of COX-2 by RNAi significantly inhibits KYSE450 cell growth in nude mice. pSilencer-COX-2 (S1 and S2 clones) and pSilencer (Vector) cells (4 x 10\textsuperscript{6}) prepared from exponentially growing cultures were subcutaneously inoculated into the right flanks of nude mice. Tumor formation was scored 45 days later. Photographs of the representative tumors removed from mice (scale = 5 cm) (D). The graph shows the tumor weights of mice injected subcutaneously with pSilencer-COX-2 or pSilencer cells after 45 days. These results are the average values of the five mice and the error bars indicate the SD. Similar results were obtained in two additional experiments. \( P < 0.05 \) compared with pSilencer control cells by Student’s t-test (E).

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References

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