Selective Inhibition of Cyclooxygenase-2 Suppresses Growth and Induces Apoptosis in Human Esophageal Adenocarcinoma Cells

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ABSTRACT

Adenocarcinoma in Barrett’s esophagus has been increasing in incidence at a rapid rate for more than two decades. Cyclooxygenase (COX)-2 appears to play an important role in gastrointestinal carcinogenesis, and COX-2 overexpression has been demonstrated both in esophageal adenocarcinomas and in the metaplastic epithelium of Barrett’s esophagus. The aim of our study was to determine whether selective inhibition of COX-2 by NS-398 would alter the rates of cell growth and apoptosis in human Barrett’s-associated esophageal adenocarcinoma cell lines. COX-1 and COX-2 expression in adenocarcinoma cell lines was determined using reverse transcription-PCR and Western blotting for mRNA and protein, respectively. Esophageal adenocarcinoma cell lines were treated with various concentrations of NS-398 (selective for COX-2 inhibition) and flurbiprofen (selective for COX-1 inhibition). Cell growth was compared in flurbiprofen-treated and untreated tumor cell lines; cell growth and apoptosis were compared in NS-398-treated and untreated tumor cell lines. COX-2 mRNA and protein were detected in two of three cell lines (SEG-1 and FLO): the third cell line, BIC-1, did not express COX-2 mRNA or protein under basal conditions or after stimulation with phorbol 12-myristate 13-acetate. Treatment with COX-1-selective concentrations of flurbiprofen did not affect cell growth in any of the three tumor cell lines. In contrast, treatment with COX-2-selective concentrations of NS-398 significantly suppressed cell growth and increased apoptosis in the cell lines that expressed COX-2 (SEG-1 and FLO), but not in the cell line that did not express COX-2 (BIC-1). We conclude that the administration of a selective inhibitor of COX-2 significantly decreases cell growth and increases apoptosis in Barrett’s-associated adenocarcinoma tumor cells that express COX-2. These observations suggest a potential role for selective COX-2 inhibitors in the prevention and treatment of esophageal adenocarcinoma for patients with Barrett’s esophagus.

INTRODUCTION

Cancer of the esophagus is one of the most lethal malignancies of the gastrointestinal tract. The dismal prognosis for patients with esophageal cancer has changed little over the past two decades, and 5-year survival rates remain well below 20% (1). During this same period, however, profound changes have been observed in the relative frequencies of the two major histological types of esophageal cancer. Since the mid-1970s, the incidence of squamous cell carcinoma of the esophagus has declined, whereas esophageal adenocarcinoma has more than tripled in frequency (2, 3). GERD (4) has been established as a strong risk factor for adenocarcinoma of the esophagus (4), and more than 40% of adult Americans experience regular GERD symptoms (5). In some individuals, the chronic esophageal inflammation induced by GERD results in intestinal metaplasia, a condition known as Barrett’s esophagus. The metaplastic epithelium is predisposed to malignancy, and most esophageal adenocarcinomas are judged to arise from Barrett’s esophagus (4, 6).

COXs are the key enzymes that mediate the production of prostaglandins from arachidonic acid. Two isoforms of COX have been identified, COX-1 and COX-2. COX-1 is expressed constitutively, whereas COX-2 can be induced by a number of agents including cytokines, growth factors, and tumor promoters (7–10). Data from both human and animal studies suggest an important role for COX-2 in gastrointestinal tumorigenesis (11, 12). Studies in vitro have shown that overexpression of COX-2 reduces the rate of apoptosis, increases the invasiveness of malignant cells, and promotes angiogenesis (13–19). Up-regulation of COX-2 has been observed in a number of human tumors including colorectal, pancreatic, and gastric adenocarcinomas (20–24). Furthermore, overexpression of COX-2 has been detected in human esophageal squamous cell carcinomas and adenocarcinomas and in the nonmalignant, metaplastic epithelium of Barrett’s esophagus (25–27).

A number of epidemiological studies have concluded that the use of aspirin and other NSAIDs that inhibit both COX-1 and COX-2 may protect against the formation of gastrointestinal tumors (28–33). Recent data suggest that this antitumor effect may be the result of inhibition of COX-2. NSAIDs that selectively inhibit COX-2 have been shown to reduce the formation of colorectal carcinomas in animal models, to inhibit the formation of colonies by human colorectal carcinoma cell lines, and to retard the growth of human pancreatic carcinoma cell lines (22, 34–37). COX-2-selective NSAIDs also have been shown to decrease both the number and size of colonic polyps in patients with familial adenomatous polyposis (12). However, the conclusions that can be drawn from these studies are limited because the investigators often used high doses of the so-called COX-2-selective NSAIDs, and this may have resulted in tissue concentrations that were no longer selective for COX-2 (i.e., COX-1 may have been inhibited as well). Furthermore, some data suggest that NSAIDs may prevent carcinogenesis through mechanisms other than COX inhibition. For example, NSAIDs that possess no COX-inhibitory activity have been shown to inhibit the growth of colon tumors both in vivo and in vitro and to inhibit the proliferation of pancreatic carcinoma cell lines (22, 38, 39). Thus, it is not clear whether the antitumor effects of NSAIDs result from inhibition of COX-1, COX-2, or both or from some COX-independent mechanism.

One recent study has shown that selective COX-2 inhibitors, used at doses that maintained their COX-2 specificity, did indeed reduce proliferation and increase apoptosis in esophageal squamous carcinoma cell lines (26). However, the effects of selective COX-2 inhibition on the growth of Barrett’s-associated esophageal adenocarcinoma cell lines have not been reported. Using appropriate doses of the COX-2-selective inhibitor NS-398 and the COX-1-selective inhibitor flurbiprofen, we have studied the effects of COX inhibition on cell growth and apoptosis in Barrett’s-associated esophageal adenocarcinoma cell lines.
RESULTS

Expression of COX-1 and COX-2 in Barrett’s-associated Esophageal Adenocarcinoma Cell Lines. COX-1 and COX-2 mRNA and protein expression was assessed in each of the three adenocarcinoma cell lines. All three esophageal adenocarcinoma cell lines (BIC-1, SEG-1, and FLO) expressed COX-1 mRNAs (Fig. 1A) and proteins (Fig. 1B); the protein levels expressed by BIC-1 were twice those expressed by SEG-1 and FLO, as measured by densitometry. There were marked differences in expression of COX-2 among the three adenocarcinoma cell lines. SEG-1 expressed high levels of COX-2 mRNA (Fig. 2A) and protein (Fig. 2B), whereas FLO showed only faint expression of COX-2 mRNA (Fig. 2A) and weak protein expression (Fig. 2B). By densitometry, SEG-1 expresses approximately five times the amount of COX-2 protein expressed by FLO. In contrast, BIC-1 did not express either COX-2 mRNA (Fig. 2A) or protein (Fig. 2B). To confirm these results and to explore further differences in COX-2 protein expression, each esophageal adenocarcinoma cell line was treated with 50 ng/ml PMA to stimulate the expression of COX-2 protein. Increased COX-2 protein was detected in both SEG-1 and FLO after PMA stimulation (Fig. 3), whereas no COX-2 protein was detected in BIC-1 after stimulation with PMA (Fig. 3).

Fig. 1. COX-1 expression in esophageal adenocarcinoma cell lines. In A, COX-1 cDNA was amplified from 5 μg of total RNA from each cell line; GADPH cDNA served as an internal control. In B, lysate protein (100 μg/lane) was loaded onto a 10% SDS gel, electrophoresed, and transferred to nitrocellulose. The blot was probed with COX-1-specific antibody.
Effect of NS-398 on Cell Growth. The effects of NS-398 on esophageal adenocarcinoma tumor cell growth were assessed. Tumor cells were treated with vehicle or NS-398 in concentrations ranging from 0.1–10 μM (a concentration range selective for COX-2), and the effects on cell growth were determined after 48 h of treatment. Compared with cells treated with vehicle only, treatment with NS-398 significantly inhibited growth of SEG-1 and FLO, whereas no significant effect at these concentrations was observed in BIC-1 (the cell line that expresses no COX-2; Fig. 4A).

Effect of Flurbiprofen on Cell Growth. The effects of flurbiprofen on esophageal adenocarcinoma tumor cell growth were assessed. Tumor cells were treated with vehicle or flurbiprofen in concentrations ranging from 0.1–5 μM (a concentration range selective for COX-1), and the effects on cell growth were determined after 48 h of treatment. Compared with cells treated with vehicle only, no significant effect of flurbiprofen at these concentrations was observed on cell growth in any of the Barrett's-associated adenocarcinoma cell lines (Fig. 4B).

Effect of NS-398 on Apoptosis. A cell death ELISA assay was used to determine whether the significant decrease in cell growth observed after treatment with NS-398 was the result of enhanced apoptosis in Barrett's-associated adenocarcinoma cell lines. Attached and unattached cells from each adenocarcinoma cell line were analyzed after 36 h of treatment with 0.1–10 μM NS-398 or vehicle control. Apoptosis was significantly increased in SEG-1 and FLO after treatment with NS-398 (Fig. 5). However, BIC-1 showed no significant increase in apoptosis when treated with these concentrations was observed on cell growth in any of the Barrett's-associated adenocarcinoma cell lines (Fig. 4B).

Effect of NS-398 on Apoptosis. A cell death ELISA assay was used to determine whether the significant decrease in cell growth observed after treatment with NS-398 was the result of enhanced apoptosis in Barrett's-associated adenocarcinoma cell lines. Attached and unattached cells from each adenocarcinoma cell line were analyzed after 36 h of treatment with 0.1–10 μM NS-398 or vehicle control. Apoptosis was significantly increased in SEG-1 and FLO after treatment with NS-398 (Fig. 5). However, BIC-1 showed no significant increase in apoptosis when treated with NS-398 (Fig. 5). As an additional assessment of apoptosis, in situ fluorescein labeling of apoptotic DNA strands was performed in all three adenocarcinoma cell lines. Cells were treated with 10 μM NS-398 or vehicle control, stained, and then examined using confocal microscopy. The dose of 10 μM NS-398 was selected because this concentration inhibits 100% of COX-2 activity but has a minimal effect on the function of COX-1. Treatment with 10 μM NS-398 caused a marked induction of apoptosis among adenocarcinoma cell lines SEG-1 and FLO. Increased nuclear staining in both SEG-1 and FLO cells treated with 10 μM NS-398 was observed compared with tumor cells treated with vehicle alone (Fig. 6, B and C). No difference in nuclear staining was observed in BIC-1 tumor cells treated with 10 μM NS-398 when compared with vehicle-treated controls (Fig. 6A).
DISCUSSION

We have shown that certain Barrett’s-associated adenocarcinoma cell lines (SEG-1 and FLO) express COX-2 mRNAs and proteins under both basal and stimulated conditions. These cell lines were used as a model to study in vitro the effects of COX-2 inhibition in Barrett’s adenocarcinoma. We have found that selective inhibition of COX-2 by NS-398 significantly decreased cell growth and increased apoptotic cell death in the cell lines that expressed COX-2. In contrast, we have found no significant growth or apoptotic effects for NS-398 in BIC-1, a cell line that did not express COX-2. Furthermore, we found no significant effect on cell growth of flurbiprofen at doses selective for COX-1 in any of the three Barrett’s-associated adenocarcinoma cell lines. These observations suggest that COX-2 inhibition plays a role in the antiproliferative and proapoptotic effects of NS-398 in Barrett’s-associated esophageal adenocarcinoma cell lines. Zimmerman et al. (26) have reported similar COX-2-dependent effects for NS-398 in esophageal squamous carcinoma cell lines.

In contrast to our findings and those of Zimmerman et al. (26), some reports have suggested that the tumor-suppressive effects of selective COX-2 inhibitors are mediated through COX-2-independent pathways (14, 22). It is possible that esophageal carcinoma cell lines differ from other carcinoma cell lines in their dependence on COX-2 for proliferation. An alternative explanation for the differences among these studies relates to the high doses of NS-398 used by the investigators. Studies on colorectal and pancreatic tumor cell lines used concentrations of NS-398 of $>10 \mu M$, whereas studies in esophageal carcinoma cells used lower concentrations of this drug (14, 22, 26). At concentrations above 10 $\mu M$, NS-398 has been shown to lose its selectivity for COX-2 (43). Thus, effects observed at these concentrations might result from inhibition of COX-1 as well as COX-2. Furthermore, the use of such high concentrations of NS-398 may affect cellular targets other than COX. He et al. (44) have shown that nonselective NSAIDs, when used in concentrations 10–20-fold higher than those required to inhibit COX activity, down-regulate tran-

Fig. 6. Dual-label fluorescence image of esophageal adenocarcinoma cells. Cells were seeded onto chamber slides and treated with 10 $\mu M$ NS-398 or vehicle for 48 h. Rhodamine phalloidin labeling of actin and fluorescein labeling of apoptotic DNA strands were then performed. Increased intensity and specificity in nuclear staining by fluorescein (white arrows) are seen after treatment with NS-398 in SEG-1 and FLO relative to cells treated with vehicle only. No difference in nuclear staining by fluorescein is observed in BIC-1 cells treated with NS-398 compared with cells treated with vehicle only.
tional activity of the PPAR δ. Conceivably, the use of very high concentrations of NS-398 might affect PPAR or other genes involved in proliferation, and such effects might account for the observed COX-2-independent actions of these drugs.

Our data suggest that the antiproliferative and proapoptotic effects of NS-398 in Barrett’s-associated esophageal adenocarcinoma cell lines are mediated, at least in part, through COX-2 inhibition and are not a consequence of the nonselective inhibition of COX-1. In all of our cell lines, the COX-1-selective inhibitor flurbiprofen had no effects on cell growth. Moreover, in the COX-2-expressing cell lines SEG-1 and FLO, 100 μM NS-398 (a concentration that inhibited both COX-1 and COX-2) did not decrease cell growth and increase apoptosis any more than 10 μM NS-398 (a COX-1-sparing concentration; data not shown). In BIC-1, a cell line that does not express COX-2, we found no significant growth or apoptotic effects for any dose of NS-398 up to 100 μM NS-398 (data not shown).

We observed a difference in susceptibility to apoptosis induced by NS-398 in SEG-1 and FLO that appeared to correlate the levels of COX-2 expression. In FLO, which expressed low levels of COX-2, NS-398 significantly inhibited apoptosis even at the lowest dose tested (0.1 μM). In SEG-1, which expressed COX-2 abundantly, significant apoptosis was observed only at a dose of 10 μM NS-398. If COX-2 is essential for the antiproliferative and proapoptotic effects of NS-398, one might expect the cell line that expresses more COX-2 to be more susceptible to the inhibitory effects of NS-398. However, recent data suggest that the alternative theory, i.e., cells that are dependent on COX-2 for growth but express low levels of COX-2 may be more susceptible to the inhibitory effects of NS-398, is also plausible (17, 45). Moreover, a difference in the baseline rate of apoptosis between the cell lines might also influence the effects of COX-2 inhibition. Using the cell death ELISA assay, we determined the baseline rate of apoptosis at 24 h in FLO and SEG-1. Indeed, the baseline rate of apoptosis was more than 3-fold higher in FLO compared with SEG-1 (data not shown). Conceivably, the lower baseline rate of apoptosis in SEG-1 may underlie its decreased susceptibility to the antiproliferative effects of COX-2 inhibition. Finally, PPAR inhibitors and other COX-2-selective inhibitors were not analyzed in this study; therefore, we cannot exclude the hypothesis that alternative effects of NS-398 (other than those mediated by COX-2) may have contributed to its proapoptotic effect and that such effects might preclude apoptosis in FLO cells.

In conclusion, we have shown that certain Barrett’s-associated esophageal adenocarcinoma cell lines express COX-2, and that treatment with a selective inhibitor of COX-2 (NS-398) significantly decreases cell growth and increases apoptosis. These results provide an experimental basis for clinical studies designed to determine whether COX-2 inhibitors will be useful in the chemoprevention or treatment of adenocarcinoma in Barrett’s esophagus.

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