Aspirin Induction of Apoptosis in Esophageal Cancer: A Potential for Chemoprevention

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Abstract
The potential use of non-steroidal anti-inflammatory drugs (NSAIDs) in the prevention of gastrointestinal cancers has been highlighted recently. However, it is not known whether NSAIDs could also be useful for preventing esophageal cancer, although regular users of these drugs appear to have a decreased incidence of esophageal cancer. Therefore, we examined the effect of aspirin on growth and apoptosis in 10 esophageal cancer cell lines as well as the expression and modulation of its target enzymes, cyclooxygenases (COXs), and their product prostaglandin E2. Growth inhibition of these cells by aspirin was dose- and time-dependent and associated with the induction of apoptosis. COX-1 and COX-2 were expressed in 7 of the 10 cell lines. Bile acids could induce COX-2 expression in six of eight cell lines tested, which was correlated with prostaglandin E2 production, and aspirin could inhibit COX-2 enzymatic activity even after bile acid stimulation but was unable to change the COX-2 protein level in these cell lines. Down-regulation of bel-2 by aspirin was found in the two cell lines tested. These results suggest that induction of apoptosis by aspirin may be a mechanism by which it can intervene in esophageal carcinogenesis and may be indicative of the potential of NSAIDs as chemopreventive agents in esophageal cancer.

Introduction
Esophageal cancer remains a significant health problem worldwide; in the United States alone, ~12,500 new cases will be diagnosed and 12,200 patients will die of the disease in 1999, which makes this cancer the seventh leading cause of cancer deaths in men in the United States (1). Epidemiological studies indicate that the incidence of esophageal adenocarcinoma is increasing in the United States and in Europe (2–5). The factors underlying this increase are unknown but may be related to Barrett’s esophagus, the presence of which is associated with a 30–125-fold increase in the risk of developing adenocarcinoma of the esophagus (2–7). The incidence of esophageal cancer differs greatly among racial groups in the United States; squamous cell carcinoma of the esophagus occurs five times more frequently in black men than in white men, but adenocarcinoma of the esophagus occurs more often in whites. The incidence of esophageal cancer among blacks also is greater at younger ages (5, 7).

Risk factors such as cigarettes and alcohol are significant for both histological types of the cancer in the United States, although higher risk is noted for adenocarcinoma in individuals who have gastroesophageal reflux, which usually results in Barrett’s esophagus, and who are in the highest decile of body mass index (5, 7). In Asian countries, however, especially in northern China, squamous cell carcinoma of the esophagus is dominant, and the risk factors are associated more with nutritional deficiencies and consumption of pickled vegetables (5, 7).

The treatment and prognosis of both types of esophageal cancer are quite similar (5). Surgical resection provides excellent palliation of the neoplasm; however, the rate of cure with esophagectomy alone is only 10–20%. Adjuvant therapy at the present time with pre- or postesophagectomy irradiation may improve local-regional control but does not improve survival (8–10). Therefore, early identification and new strategies for treatment of esophageal carcinoma are urgently needed. One possible approach is to use NSAIDs3 because recent epidemiological and experimental studies have demonstrated the therapeutic potential of NSAIDs in the chemoprevention of esophageal cancer (11–13).

The effects of NSAIDs are thought to be mediated mainly through the inhibition of COXs, which are the key enzymes in the biosynthesis of prostaglandins (prostanoids) through the conversion of arachidonic acid to prostaglandin H2, the precursor of prostanooids (14). COX-1, the first cloned isozyme, is constitutively expressed in many tissues and is thought to be involved in the homeostasis of various physiological functions; another isozyme, COX-2, is elevated in tumors, including esophageal cancer, and is also inducible by various agents such as growth factors and tumor promoters (14). Tsujii and DuBois (15) reported that intestinal epithelial cells overexpressing the COX-2 gene exhibited altered adhesion properties and resisted apoptosis. Because these changes were reversed by treatment with NSAIDs, it was suggested that overexpression of COX-2 may be responsible for colorectal carcinogenesis. Other recent studies indicated that apoptosis induced by sulindac metabolites in colorectal cancer cells was independent of COX inhibition or

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3 The abbreviations used are: NSAIDs, non-steroidal anti-inflammatory drugs; COX, cyclooxygenase; FBS, fetal bovine serum; PGE2, prostaglandin E2; CD, chenodeoxycholate; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling.
Most NSAIDs research to date has focused on colon cancer, and the effects of NSAIDs on esophageal cancer cell lines in vitro have not yet been reported. Therefore, we like to investigate their potentials for chemoprevention or therapy of esophageal cancer.

In this study, we tested the effect of aspirin on 10 esophageal cancer cell lines, analyzed the expression of COX-1 and -2 in these cell lines, examined the stimulation of COX-2 by bile acids, and assessed PGE2 production. In addition, we also examined the expression of apoptosis-related genes in these cells.

Materials and Methods

Cell Lines. Esophageal cancer cell lines of the TE and SKGT series were obtained from the First Department of Pathology, Hiroshima University School of Medicine, Hiroshima, Japan, and Surgery Branch, National Cancer Institute, Bethesda, MD, respectively. TE-1 and TE-3 were from well-differentiated, TE-8 and TE-12 were from moderately differentiated, and TE-2 and TE-13 were from poorly differentiated squamous cell carcinomas. SKGT-4 was from a well-differentiated adenocarcinoma arising in Barrett's esophagus, and TE-7, SKGT-5, and BE-3 were from poorly differentiated adenocarcinomas.

Cell Culture and Treatment with Aspirin and Bile Acids. The above-named cell lines were plated in tissue culture dishes and grown in DMEM with 10% FBS at 37°C in a humidified atmosphere of 95% air and 5% CO2. To examine the effect of aspirin, the cells were plated in regular medium and incubated for 24 h; the medium was then replaced with either control medium or with medium containing aspirin at 1, 3, or 5 mM. The aspirin was dissolved in DMSO and diluted into the medium before each experiment. The medium was completely replaced with fresh medium every 72 h. At the end of the experiment, the cells were fixed with 10% trichloroacetic acid, stained with 0.4% sulforhodamine B in 1% acetic acid, and the absorbances were determined using an automated spectrophotometric plate reader at a single wavelength of 490 nm. Viability was tested by exclusion of trypan blue (0.1%), and the percentage of growth inhibition was calculated by the equation: % control = (ODt/ODc × 100; where ODt and ODc are the absorbances in treated cultures and control cultures, respectively.

To examine the modulation of COX expression by bile acids, CD and deoxycholate (Sigma Chemical Co., St. Louis, MO) were used to treat the esophageal cancer cells for 12 h at a concentration 400 μM. Cellular proteins were isolated in lysis buffer containing 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 10 μg/ml phenylmethylsulfonyl fluoride, 30 μg/ml aprotinin, and 50 mM Tris-HCl (pH 8.0). The samples were then put on ice for 60 min and centrifuged at 13,000 rpm for 30 min. Protein concentrations were measured using a Bio-Rad DC kit (Bio-Rad Laboratories, Hercules, CA), according to the manufacturer’s protocol.

DNA Extraction and Gel Electrophoretic Analysis of DNA Fragmentation. Soluble DNA was extracted after a 2-day treatment with 5 mM aspirin. The cells floating in medium were collected by centrifugation, and the cells that remained attached to the dish were detached by scraping. The cells were centrifuged into a pellet and resuspended in Tris-EDTA buffer (pH 8.0). The plasma membrane of the cell was lysed in 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100 on ice for 15 min. The lysate was centrifuged at 12,000 × g for 15 min to separate soluble (fragmented) from pellet (intact genomic) DNA. Soluble DNA was treated with RNase A (50 μg/ml) at 37°C for 1 h, followed by treatment with proteinase K (100 μg/ml) in 0.5% SDS at 50°C for 2 h. The residual material was...
extracted with phenol-chloroform, precipitated in ethanol, electrophoresed on a 1.8% agarose gel, and stained with ethidium bromide. The gels were then photographed in the dark using UV illumination.

**TUNEL Assay.** The TUNEL assay was performed with a commercial kit (APO-BRDU Apoptosis Kit; Phoenix Flow Systems, San Diego, CA). The esophageal cancer cells were treated with control medium or medium containing 5 mM aspirin for 2 or 5 days. Both floating and adherent cells were collected, labeled with fluorescein dye, and stained with propidium iodide according to a protocol provided by the manufacturer. The cells were then analyzed by flow cytometry using a FACScan flow cytometer (Epics Profile; Coulter Corp., Hialeah, FL).

**Western Blotting.** Samples containing 30 μg of protein extracted from either control or treated esophageal cancer cells were subjected to gel electrophoresis in 10–14% polyacrylamide slab gels in the presence of SDS (SDS-PAGE). The proteins were then transferred electrophoretically to a Hybond-C nitrocellulose membrane (Amersham, Arlington Heights, IL) at 150 V for 1 h at 4°C. The membrane was subsequently immersed in 0.5% Ponceau S in 1% acetic acid to stain the proteins and to verify that equal amounts of protein were loaded in each lane and transferred efficiently. After incubating overnight in a blocking solution containing 15% bovine skim milk in 10 mM PBS (pH 7.4), the nitrocellulose membranes were incubated for 3 h with primary antibodies, i.e., anti-COX-1 from Cayman Chemicals (Ann Arbor, MI), COX-2 and bcl-2 from Transduction Laboratories (Lexington, KY), bax and p21 from Oncogene (Cambridge, MA), or β-actin from Sigma. The membranes were then washed with PBS buffer to remove excess unbound antibodies and incubated for 1 h with horse antimouse or goat antirabbit secondary antibody (Amersham) at 1:2000. After incubation, the membranes were washed in PBS containing 0.1% Tween-20, incubated with ECL solution (Amersham) for 1–2 min, and exposed to an X-ray film for chemiluminescence.

**ELISA Assay of PGE2 Production.** Cells (2 × 10⁴/well) were plated in 24-well dishes and grown to 80% confluence in DMEM containing 10% FCS. The medium was then replaced with DMEM containing 1% FCS and vehicle (DMSO), or aspirin (5 mM), CD (400 μM) or CD plus aspirin (aspirin pretreatment for 8 h) for 12 h. The conditioned medium was then collected to determine the production of PGE2 by a commercial kit (Cayman) according to the manufacturer’s protocol.

### Results

**Decrease in Cell Numbers by Aspirin Was Due to Induction of Apoptosis.** To determine the effect of aspirin on the growth of esophageal cancer cell lines, the cells in monolayer culture were treated with 1, 3, or 5 mM aspirin for 1, 3, 5, or 7 days, respectively (Fig. 1). Maximal effects were reached when these cells were treated with 5 mM aspirin for 7 days. The sensitivities of the different cell lines varied considerably. DNA fragmentation analysis revealed that decreased growth of esophageal cancer cells was due to induction of apoptosis by aspirin (Fig. 2), and flow cytometry data showed that these cells underwent apoptosis after 2 or 5 days of treatment with 5 mM aspirin (Table 1).

**Expression of COX-1 and COX-2 and Modulation by Bile Acids.** To determine whether the effect of aspirin is associated with COX expression, COX protein was first determined in these cell lines by Western blotting (Fig. 3). Seven cell lines...
expressed both COX-1 and COX-2 at different levels when grown in monolayer cultures in medium supplemented with 10% FBS, whereas TE-1, SKGT-5, and BE-3 cells failed to express either isozyme, and SKGT-4 expressed only a low level of COX-2 (Fig. 3). A comparison of cell growth (Fig. 1) with COX expression (Fig. 3) showed no significant relationship, but it appeared that cells that expressed COX-1 and -2, except TE-3, were more sensitive to aspirin.

Bile acids, putative tumor promoters in gastrointestinal carcinogenesis, were able to induce COX-2 but not COX-1 expression in six of eight cell lines tested (Fig. 4). PGE2 production was also increased by severalfold after bile acid stimulation in two of three cell lines tested (Fig. 5).

Inhibition of PGE2 Production by Aspirin. To investigate the correlation of COX expression with PGE2 production, we selected three esophageal cancer cell lines that express high, low, or no COX-2 protein, respectively, and then measured the PGE2 level, its modulation by bile acids, and its inhibition by aspirin, using an ELISA assay and Western blotting. Fig. 5B shows that TE-1 cells, which do not express either COX-1 or COX-2 and are not modulated by bile acids (see Figs. 4 and 5B), had almost no PGE2 in the conditioned medium, whereas TE-7 and SKGT-4 cells, which expressed both COX-1 and COX-2 and produced increased amounts of COX-2 protein after treatment with bile acids, produced a large amount of PGE2 (Fig. 5). Aspirin was able to block PGE2 production, even after bile acid stimulation, but was unable to change expression of COX-2 protein in these cell lines (Fig. 5A).

Modulation of bcl-2 by Aspirin. To study the effects of aspirin on apoptosis-related genes, we selected two cell lines, one expressing high levels of COX-2 (TE-7) and another one showing no COX-2 protein by Western blotting (TE-1), and then analyzed the expression of bcl-2, bax, and p21 proteins by Western blotting. The data showed that aspirin was able to reduce bcl-2 protein expression after as little as 6 h of treatment (Fig. 6), whereas there were no changes in expression of bax (data not shown) and p21 (Fig. 6) proteins.

Discussion

This study is the first detailed investigation of the effect of aspirin on esophageal cancer cell lines, although several recent studies have shown that NSAIDs can inhibit the growth of different cancer cells, including glioma and colorectal and gastric cancers (16–20). Our data demonstrate that aspirin inhibits the growth and induces apoptosis of esophageal squamous cell carcinoma and adenocarcinoma cell lines. Bile acids, putative tumor promoters (21), were able to induce COX-2 expression and PGE2 production, which could be blocked by aspirin. Regardless of COX expression, aspirin could reduce bcl-2 protein expression after as little as 6 h of treatment (Fig. 6), whereas there were no changes in expression of bax (data not shown) and p21 (Fig. 6) proteins.

COX-2 and -2 expression by bile acids. Esophageal cancer cell lines were grown on monolayer cultures in DMEM with 10% FBS and with or without bile acids for 12 h. Total cellular protein was extracted, and the levels of COX-1 and -2 were determined by Western blotting. C, control (no bile acids); CD, chenodeoxycholate; DC, deoxycholate.

Fig. 4. Modulation of COX-1 and -2 expression by bile acids. Esophageal cancer cell lines were grown on monolayer cultures in DMEM with 10% FBS and with or without bile acids for 12 h. Total cellular protein was extracted, and the levels of COX-1 and -2 were determined by Western blotting. C, control (no bile acids); CD, chenodeoxycholate; DC, deoxycholate.

Fig. 5. ELISA analysis of PGE2 production with or without aspirin and bile acid treatment in esophageal cancer cells. TE-1, TE-7, and SKGT-4 cell lines were selected based on no, high, and low expression of COX-2, respectively (see A for TE-7 and SKGT-4 and Figs. 3 and 4 for TE-1). The cells were grown in monolayer cultures for 12 h, and the conditioned media were then subjected to ELISA assay for detection of PGE2 production (B).

Fig. 6. Western blot analysis of apoptosis-related gene expression in esophageal cancer cells. The cells were grown in monolayers without (C) and with aspirin for 3, 6, 12, or 24 h, respectively. The cellular protein was then extracted and blotted with antibody (see “Materials and Methods”).
most cell lines, COX expression seemed to indicate sensitivity to aspirin. There were, however, exceptions; TE-1 cells, which do not express COX-1 or COX-2, showed an ~50% decrease in cell numbers after a 7-day treatment with 5 mm aspirin, whereas TE-3 cells, which express both COX-1 and COX-2, were relatively resistant to aspirin treatment. Previous studies demonstrated that the induction of apoptosis by NSAIIDs was independent of COX inhibition, cell cycle arrest, or p53 induction (16, 17). This study shows that aspirin can reduce bcl-2 expression after as little as 6 h of treatment in two esophageal cancer cell lines tested and that this effect is independent of COX expression of the cell lines. Sheng et al. (22) recently reported that PGE2 was able to induce bcl-2 expression in colon cancer cell lines, which can only partially explain the mechanism by which aspirin induces apoptosis in cancer cells because in this study aspirin down-regulated bcl-2 expression in TE-1 cells, which do not express COX-1 or COX-2 and produce only very low levels of PGE2. Again, two studies did demonstrate that COX-2-selective analogues could inhibit colon carcinogenesis (23, 24). A different signaling pathway may be involved in the action of aspirin, which provides insight into the induction of apoptosis by NSAIIDs. For example, aspirin exhibited anti-activator protein activity and inhibited nuclear factor-κB (25, 26). Therefore, the mechanisms by which NSAIIDs prevent cancer and induce apoptosis in neoplastic cells are not that simple and need further study.

Analysis of COX-2 protein in surgical specimens by immunohistochemistry demonstrated that this isozyme is overexpressed in esophageal cancer cells, whereas normal esophageal epithelium expressed no or weak COX-2 (27, 28). In this study, COX-2 was overexpressed in 7 of 10 esophageal cancer cell lines, whereas normal esophageal epithelium expressed no or weak COX-2 (27, 28). Therefore, the mechanisms by which NSAIIDs prevent cancer and induce apoptosis in neoplastic cells are not that simple and need further study.

Nevertheless, the numerous side-effects of aspirin in the gastroenterological tract may prevent patients from long-term use, although the dose used in the present study is clinically achievable. At the present time, a few potent NSAIIDs with fewer side-effects are available and indeed are being used in clinical prevention trials for different cancers.

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References

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