Induction of Apoptosis in Colon Cancer Cells by Cyclooxygenase-2 Inhibitor NS398 through a Cytochrome c-dependent Pathway

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ABSTRACT
Nonsteroidal anti-inflammatory drugs (NSAIDs) have shown cancer preventive activity in patients who took them frequently. These drugs can induce tumor cells to undergo apoptosis in vitro. NS398, a cyclooxygenase-2 (COX-2)-selective inhibitor, has been reported to cause apoptosis in cancer cell lines. Therefore, we examined its effect on 15 human colon cancer cell lines and investigated its mechanism of action. NS398 decreased cell viability in all of the cell lines. Tumor cells that expressed COX-2 were shown to be more sensitive to NS398 treatment. In three selected colon cancer cell lines, NS398-induced apoptosis was mediated by the release of cytochrome c from mitochondria and, consequently, by the activation of caspase-9 and caspase-3 and by the cleavage of poly(ADP-ribose) polymerase. In contrast, caspase-8 was not involved in NS398-induced apoptosis, which suggested that the cytochrome c pathway may play an important role in NS398-induced apoptosis in colon cancer cell lines. Therefore, the combination of NS398 with apoptosis-inducing drugs through cytochrome c-independent pathways may be warranted.

INTRODUCTION
Improvements in surgical and adjuvant therapies and in dietary and screening programs have facilitated an overall decline in the mortality of colon cancer. Nevertheless, colorectal cancer still accounts for 11% of all cancers in the United States with an estimated 130,200 new cases and 56,300 deaths expected in the year 2000 (1). In recent years, several important advances have been made in understanding the biology and genetics of colorectal cancer, and new evidence has linked the use of NSAIDs with the decreased incidence of colorectal cancer (2–8). Epidemiological studies showed that the rate of mortality from colorectal cancer in individuals taking NSAIDs was 40–50% lower than that in nonusers. Among patients with familial adenomatous polyposis, treatment with sulindac or celecoxib significantly reduces the size and number of adenomas (9, 10). In animal models, NSAIDs can inhibit colorectal carcinogenesis, which can reduce the frequency and number of premalignant and malignant lesions (11–13). On the basis of accumulated data, several clinical cancer prevention trials using NSAIDs are in progress.

COX-2 is frequently overexpressed in the cells of various tumors (7, 8, 14–17). Thus, neoplastic and nonneoplastic cell proliferation and immune functions may be controlled in part by endogenous prostaglandin synthesis, a process that is inhibited by NSAIDs. However, the mechanism by which NSAIDs prevent cancer and induce apoptosis in neoplastic cells is not yet clear (18). Apoptosis is a tightly regulated process involving changes in the expression or activities of distinct genes (19). Dozens of such genes involved in the induction or inhibition of apoptosis have been cloned and analyzed and major apoptotic pathways (e.g., Fas, p53, and cytochrome c pathways) have been identified (19).

In this study, we investigated the possible mechanisms of NS398’s action in colon cancer cell lines. Our results show that NS398 decreased tumor cell viability and that the COX-2-independent effect did exist, although there was a positive correlation of COX-2 expression with the sensitivity to NS398 (P = 0.03 at the dose of 100 μM). The effect of NS398 was mediated by the release of cytochrome c from mitochondria and, consequently, by the activation of caspase-9 and caspase-3 and by the cleavage of PARP. However, our data indicated that caspase-8 is not involved in NS398-induced apoptosis.

MATERIALS AND METHODS
Cell Culture and Treatment. The human colon cancer cell lines GEO, LS174, MIP101, OMEGA, RKO, SW403, SW480, SW620, C-cell, DIFI, DLD-1, HT-29, HCT-15, KM12LI, and KM12SW were plated in tissue culture dishes and grown in DMEM with 10% fetal bovine serum at 37°C in a humidified atmosphere of 95% air and 5% CO2. To evaluate the effect of NS398, the cells were plated in DMEM for 24 h. The medium was then replaced either with control medium containing 0.01% DMSO or with medium containing 50 or 100 μM NS398 (Biomol Research Laboratories, Inc., Plymouth Meeting, PA) dissolved in DMSO (stock solution of 100 mM) and diluted in the medium before each experiment. At the end of the experiment, the cells were fixed with 10% trichloroacetic acid (TCA) and stained with 0.4% SRB in 1% acetic acid. The unit of absorbance was then read using an automated spectrophotometric plate reader at a single wavelength of 490 nm. Cell viability was tested by exclusion of trypan blue (0.1%). The percentage of control was determined using the following equa-

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3 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; PARP, poly(ADP-ribose) polymerase; SRB, sulforhodamine B; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

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tion: \( \% \text{ growth inhibition} = (1 - A_t/A_c) \times 100 \), where \( A_t \) and \( A_c \) represent the unit of absorbance in the treated and control cultures, respectively.

**DNA Fragmentation Assay.** Soluble DNA was extracted from both floating and attached cells after a 2-day treatment with 100 \( \mu \)M NS398. Briefly, the cells were pelleted by centrifugation and resuspended in Tris-EDTA buffer (pH 8.0). The plasma membrane of the cell was lysed on ice in a mixture of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5% Triton X-100 for 15 min. The lysate was centrifuged at 12,000 \( \times \) g for 15 min to separate soluble (fragmented) from pellet (intact genomic) DNA. Soluble DNA was treated with RNase A (50 \( \mu \)g/ml) at 37°C for 1 h, followed by treatment with proteinase K (100 \( \mu \)g/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 under UV illumination.

**TUNEL Assay.** TUNEL assay was performed using an APO-bromodeoxyuridine apoptosis kit (Phoenix Flow Systems, San Diego, CA). Briefly, the cells were treated for 2 days with medium containing 0.01% DMSO (control) or 100 \( \mu \)M NS398. The cells were then labeled with fluorescein dUTP and stained with propidium iodide according to the manufacturer’s protocol. Thereafter, the cells were analyzed for apoptosis using a FACScan flow cytometer (Epics Profile; Coulter Corp., Hialeah, FL).

**Measurement of Cytochrome c Release Using Western Blotting.** After the cells were exposed to 100 \( \mu \)M NS398 for 3–24 h, both floating and attached cells were washed with PBS (pH 7.2) and buffer A [0.25 M sucrose, 30 mM Tris-HCl (pH 7.9), and 1 mM EDTA], and pelleted by brief centrifugation. The pellets were resuspended in buffer B (buffer A plus the following protease inhibitors: 1 mM phenylmethysulfonyl fluoride, 1 \( \mu \)g/ml leupeptin, 1 \( \mu \)g/ml pepstatin, and 1 \( \mu \)g/ml aprotinin) and homogenized with a glass dounce homogenizer with a pestle (40 strokes). After centrifugation at 14,000 rpm for 30 min, the supernatants were collected, and the protein concentration was determined by using a protein assay kit (Bio-Rad, Hercules, CA). To detect cytochrome c release into the cytosol, Western blotting was performed using affinity-purified anti-cytochrome c antibody (PharMingen, San Diego, CA) diluted 1:2000 in 1% (w/v) dried milk in PBS.

**Measurement of Caspase-3 and Caspase-9 Activity.** The cells were plated onto 10-cm-diameter dishes 1 day before treatment. After the cells were exposed to 100 \( \mu \)M NS398 for 2 days, both floating and attached cells were harvested and counted. Cells (1 \( \times \) 10\(^6\)) were analyzed for caspase-3 (CPP32) and caspase-9 activity using the ApoAlert CPP32 fluorescent assay kit (Clontech, Palo Alto, CA) and caspase-9 substrate (Biomol), respectively, according to the manufacturers’ protocols. The experiments were performed in triplicate, and the results were calculated as the mean \( \pm \) SD.

**Fig. 1** Cell viability assay and expression of COX protein. A, in the cell viability assay, colon cancer cell lines were grown in a monolayer for 5 days with 0.01% DMSO (control) or with 50 \( \mu \)M or 100 \( \mu \)M NS398. The cells were fixed with TCA and stained with SRB. Absorbance was read on an automated spectrophotometric plate reader at 490 nm and percentage of growth inhibition (GI) was calculated. The experiments were in triplicate and repeated once. B, results of quantitative analysis of COX-2 protein from Western blotting using the IS-1000 digital-imaging system (Alpha Innotech Corp., San Leandro, CA). C, COX-1 and COX-2 were analyzed by Western blotting and repeated once. PC, positive control.
Protein Extraction and Western Blotting. Cellular proteins from both floating and attached colon cancer cells 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 placed on ice for 60 min and centrifuged at 14,000 rpm for 30 min. Protein concentration in the samples was measured using a protein assay kit (Bio-Rad). Samples containing 30 μg of protein extracted from either control or treated cells were subjected to SDS-PAGE using 8–15% (depending on protein size to be analyzed) denaturing polyacrylamide slab gels. The proteins were then transferred electrophoretically to a Hybond-C nitrocellulose membrane (Amersham-Pharmacia Biotech, Inc., Piscataway, NJ) at 150 V for 1 h at 4°C. To stain the proteins and to validate that equal amounts of protein were loaded in each lane and transferred efficiently, the membrane was immersed in 0.5% Ponceau S (Sigma Chemical Co., St. Louis, MD) in 1% acetic acid. After the nitrocellulose membranes were incubated overnight in a blocking solution containing 15% bovine skim milk in 10 mM PBS, the membranes were incubated for 3 h with anti-COX-1 (Cayman Chemicals, Ann Arbor, MI), anti-COX-2 (Transduction Laboratories, Lexington, KY), anti-PARP (Roche Molecular Biochemicals, Indianapolis, IN), or anti-β-actin (Sigma) antibody, and then with a second antibody from the Amersham-Pharmacia electrochemiluminescence (ECL) kit. After this incubation, the membranes were washed three times in PBS containing 0.1% Tween 20, incubated in ECL solution (Amersham-Pharmacia) for 1–2 min, and exposed to X-ray film for chemiluminescence detection of positive protein band.

RESULTS

Decreased Cell Viability by NS398 and Expression of COX in Colon Cancer Cells. NS398, N-[2-(cyclohexyloxy)-4-nitrophenyl] methanesulfonamide, is a COX-2-selective inhibitor. Cellular proteins from both floating and attached colon cancer cells 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 placed on ice for 60 min and centrifuged at 14,000 rpm for 30 min. Protein concentration in the samples was measured using a protein assay kit (Bio-Rad). Samples containing 30 μg of protein extracted from either control or treated cells were subjected to SDS-PAGE using 8–15% (depending on protein size to be analyzed) denaturing polyacrylamide slab gels. The proteins were then transferred electrophoretically to a Hybond-C nitrocellulose membrane (Amersham-Pharmacia Biotech, Inc., Piscataway, NJ) at 150 V for 1 h at 4°C. To stain the proteins and to validate that equal amounts of protein were loaded in each lane and transferred efficiently, the membrane was immersed in 0.5% Ponceau S (Sigma Chemical Co., St. Louis, MD) in 1% acetic acid. After the nitrocellulose membranes were incubated overnight in a blocking solution containing 15% bovine skim milk in 10 mM PBS, the membranes were incubated for 3 h with anti-COX-1 (Cayman Chemicals, Ann Arbor, MI), anti-COX-2 (Transduction Laboratories, Lexington, KY), anti-PARP (Roche Molecular Biochemicals, Indianapolis, IN), or anti-β-actin (Sigma) antibody, and then with a second antibody from the Amersham-Pharmacia electrochemiluminescence (ECL) kit. After this incubation, the membranes were washed three times in PBS containing 0.1% Tween 20, incubated in ECL solution (Amersham-Pharmacia) for 1–2 min, and exposed to X-ray film for chemiluminescence detection of positive protein band.

Statistical Analysis. Correlation and regression analysis was performed to test the correlation between COX-2 expression and the sensitivity of colon cancer cells to NS398. P was generated using Statistica version 3.0a for a Macintosh computer (StatSoft, Tulsa, OK).
NSAID and has shown greater potent effects and fewer side effects than non-COX selective NSAIDs (20–22). To determine its effect on colon cancer cells, we cultured 15 human colon cancer cell lines in a monolayer and treated them with NS398 (50 or 100 μM) for 2–5 days. After being treated with 50 μM NS398 for 5 days, some of these cell lines showed a significant decrease in viability, as measured by the SRB assays, whereas others were less sensitive to NS398 (Fig. 1). A 5-day treatment with 100 μM NS398 decreased cell viability by 38–75%. We then analyzed COX expression in these cell lines. The relative levels of COX-2 protein were quantitated and are shown in Fig. 1B. OMEGA cells expressed the highest level of COX-1; SW620 cells expressed the highest level of COX-2; SW480 and DLD-1 cells expressed the lowest levels of both COX-1 and COX-2 proteins. In comparison of COX-2 expression with the decrease in cell viability, tumor cells with COX-2 expression were shown to be more sensitive to NS398 treatment, and correlation and regression analysis demonstrated $P = 0.0028$ ($r$, 0.71) and $P = 0.03$ ($r$, 0.56) in 50 μM and 100 μM of NS 398 treatment, respectively. However, a COX-2-independent effect does exist because the dose needed for growth inhibition of colon cancer cell is much higher than the one to inhibit COX-2 enzymatic activity (21).

Decrease in Cell Viability in Colon Cancer Cells Attributable to Apoptosis. To investigate the association of the sensitivity of colon cancer cell lines to NS398 with the expression of COX-2, we chose SW620, SW480, and HCT-15 cell lines, dependent on COX-2 expression (the highest, lowest, and average levels, respectively). The DNA fragmentation assays showed that all of the three cell lines underwent apoptosis after a 2-day treatment with 100 μM NS398 (Fig. 2A), but quantitative measurement of apoptosis by TUNEL assays demonstrated that the percentage of cells that underwent apoptosis after treatment with NS398 correlated with COX-2 expression: SW620 had the highest rate of apoptosis, whereas SW480 had the lowest rate at 2 days (Fig. 2B).

Cytochrome c Release from the Mitochondria as an Early Event. The release of cytochrome $c$, one of the most important respiratory-chain proteins located in the mitochondria, into the cytosol is the hallmark of apoptosis of cells treated with certain apoptosis inducers (23, 24). We, therefore, measured the cytosolic cytochrome $c$ using Western blotting in HCT-15, SW480, and SW620 cells that had been treated for up to 24 h with 100 μM NS398. As shown in Fig. 3, the levels of cytochrome $c$ in the cytosol were elevated in HCT-15 and SW620 cells after a 6-h treatment with NS398, and the levels were further increased by 24 h. In contrast, SW480 cells showed a lower and slower rate of release of cytochrome $c$ into the cytosol during NS398 treatment. In contrast to the DNA fragmentation and TUNEL assays, Western blotting showed that cytochrome $c$ release from the mitochondria was very rapid and is an early event in NS398-induced apoptosis in HCT-15 and SW620 cells. However, bcl-2, one of stimulators that promotes cytochrome $c$ release from mitochondrion, did not show any changes after NS398 treatment (data not shown).

Caspase Activation and PARP Cleavage by NS398. Cytochrome $c$ release into the cytosol is the primary event during the activation of cytochrome $c$-dependent apoptosis. However, a recent study indicated that the blockage or absence of caspase activation is sufficient to inhibit effective apoptosis (25). Moreover, caspase-9 activation has been thought to be the step immediately following cytochrome $c$ release from the mitochondria (19, 26). We, therefore, measured caspase-9 and caspase-3 activities and PARP cleavage in cytosolic extracts from HCT-15, SW480, and SW620 cells treated with 100 μM NS398. Fig. 4A shows that caspase-9 activity was increased at 24 h in all of the cell lines treated with NS398, but that SW480 showed a low level of caspase-9 activation. Caspase-3 activity was also elevated in these cells after a 24-h treatment with NS398 (Fig. 4B). Compared with the other two cell lines, SW480 showed less activation of caspase-3 (Fig. 4B) and a lower rate of PARP cleavage (Fig. 5A); the latter is one of the substrates of caspase-3 proteolysis. Using Western blotting, we detected a $M_r$ 89,000 fragment cleaved from the $M_r$ 13,000 PARP after 24-h treatment of these cells with 100 μM NS398.
DISCUSSION

We used the COX2-selective inhibitor NS398 to treat 15 colon cancer cell lines and demonstrate that NS398-induced apoptosis in colon cancer cells occurs through a cytochrome c-dependent pathway wherein cytochrome c is released from the mitochondria; caspase-9, caspase-3, and PARP enzymatic activities are elevated; and cells undergo apoptosis. Colorectal cancer is the second most common cause of cancer death in the United States (1). Both environmental and genetic factors influence the risk of colon cancer. Epidemiological and experimental evidence suggests that the use of NSAIDs reduces the risk of colorectal cancer (3, 4, 6, 9, 11–13, 27). Clinical trials have showed that the use of NSAIDs by patients with familial adenomatous polyposis significantly reduces the number and size of colorectal polyps (9, 10). However, the molecular mechanisms responsible for the chemopreventive action of these drugs has not been clearly established (18). Recent studies have demonstrated that NSAIDs can also induce tumor cells to undergo apoptosis (27–34), but the molecular pathway of NSAID-induced apoptosis is unclear.

It was recently discovered that mitochondria contain and release proteins such as cytochrome c that are involved in the apoptotic cascade (23, 24, 35–39). Cell-free systems demonstrate that mitochondrial products are rate limiting for the activation of caspases and endonucleases in cell extracts (23). Functional studies indicate that drug-induced opening or closing of the mitochondrial megachannel (permeability transition pore) can induce or prevent apoptosis. The anti-apoptotic oncoprotein bcl-2 acts on mitochondria to stabilize membrane integrity and to prevent the opening of the megachannel (39–41). These experiments indicate that cytochrome c is a key factor in apoptosis and that its release further activates caspases, resulting in the appearance of apoptosis. Our study confirms that cytosolic cytochrome c was increased in HCT15 and SW620 cells as early as 6 h after treatment with NS398 and was independent of bcl-2; thereafter, caspase-9, caspase-3, and PARP were further activated, leading in turn to the degradation of DNA into nucleosomal fragments. To induce apoptosis, p53, Fas, tumor necrosis factor α, and death receptor 5 use a different pathway distinct from that of cytochrome C. In this alternative pathway, caspase-8 proenzyme is activated and, in turn, activates caspase-3 to trigger apoptosis (19). Unlike caspase-9 and caspase-3, caspase-8 was not involved in NS398-induced apoptosis in colon cancer cells in our study.

Several studies have reported the overexpression of the COX-2 enzyme in human cancers, including colorectal cancer. Furthermore, an elevated level of COX-2 expression associated with poor prognosis of cancer patients (7, 8, 14 and our unpublished data). The overexpression of COX-2 in intestinal epithelial cells causes phenotypic changes that may enhance the tumorigenic or metastatic potential of colorectal cancer cells (42, 43). Oshima et al. (44) demonstrated that COX-2-null mutation dramatically reduced the number and size of the intestinal polyps in mice and that a novel COX-2 inhibitor was more effective than sulindac in reducing the number of polyps in APC d716 mice. These results provide evidence that COX-2 is important in tumorigenesis and indicate that COX-2-selective inhibitors may be a novel class of therapeutic agents for colorectal polyposis and cancer (44). However, several other studies have shown that there is no correlation between COX-2 expression and apoptosis induced by NSAIDs, including NS398 (16, 29, 31). The present data indicate that there is more sensitivity to NS398 treatment in the cells with COX-2 expression, but the dose was much higher than the one needed for the inhibition of COX-2, which indicates that COX-2-independent actions of NS398 do exist. Recent studies demonstrated that the induction of apoptosis by NSAIDs may be through 15-lipoxygenase-1 (45), arachidonic acid (46), or PPARδ (47). Cao et al. (46) found that exogenous arachidonic acid can cause apoptosis in colon cancer and other cell lines. The inhibition of COX-2 by NS398 should result in the accumulation of arachidonic acid in cancer cells and, therefore, would trigger apoptosis. Furthermore, 15-lipoxygenase-1 is usually reduced in colorectal cancer,
and the induced expression of 15-lipoxygenase-1 by NS398 in colon cancer cells may lead to the increase in its product 13,14-dihydroxyoctadecadienoic acid; the latter can induce colon cancer cells to undergo apoptosis (45). In addition, APC protein can inhibit PPARγ in colorectal cells, and APC gene mutation is the major cause of colorectal carcinogenesis. NSAIDs can inhibit PPARγ and may further inhibit colorectal carcinogenesis (47). Taken altogether, the mechanisms by which NSAIDs induce cancer cells to apoptosis can be COX-2 dependent or independent and several mechanistic possibilities may mediate NSAIDs’ action on the induction of apoptosis and the prevention of human cancers. However, the exact molecular signaling pathway that causes cytochrome c release from the mitochondria after NS398 treatment needs further investigation.

In summary, this study demonstrates that NS398 can induce colon cancer cells to undergo apoptosis, which occurs through the cytochrome c-dependent pathway. These findings suggest that the combination of NS398 with apoptosis-inducing drugs whose mechanisms of action follow cytochrome c-independent pathways (e.g., ceramide) should have synergistic or additive effects.

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