

Cyclooxygenase-2 Overexpression Reduces Apoptotic Susceptibility by Inhibiting the Cytochrome *c*-dependent Apoptotic Pathway in Human Colon Cancer Cells¹

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ABSTRACT

The *cyclooxygenase-2* (*COX-2*) gene encodes an inducible enzyme that converts arachidonic acid to prostaglandins and is up-regulated in colorectal neoplasms. Evidence indicates that *COX-2* may regulate apoptosis and can influence the malignant phenotype. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit *COX* enzymes and induce apoptosis in colorectal cancer cell lines, which may contribute to their antitumor effects. To determine whether forced *COX-2* expression modulates susceptibility to drug-induced apoptosis, HCT-15 colon carcinoma cells were stably transfected with the *COX-2* cDNA, and two clones overexpressing *COX-2* were isolated. Selective *COX-2* (NS398) and nonselective (sulindac sulfide) *COX* inhibitors, as well as 5-fluorouracil (5-FU), induced apoptosis (terminal deoxynucleotidyl transferase-mediated nick end labeling in a dosage-dependent manner). Forced *COX-2* expression significantly attenuated induction of apoptosis by all three of the drugs compared with parental HCT-15 cells. NSAIDs and 5-FU induced the mitochondrial release of cytochrome *c* as well as caspase-3 and -9 activation, and to a much lesser extent, caspase-8. *COX-2*-overexpressing cells showed reduced cytochrome *c* and caspase activation, relative to parental cells. A specific inhibitor of caspase-3 restored cell survival after drug treatment. *COX-2* transfectants were found to overexpress the antiapoptotic Bcl-2 mRNA and protein relative to parental cells. In conclusion, forced *COX-2* expression significantly attenuates apoptosis induction by NSAIDs and 5-FU through predominant inhibition of the cytochrome *c*-dependent apoptotic pathway. *COX-2*-mediated up-regulation of Bcl-2 suggests a potential mechanism for reduced apoptotic susceptibility.

INTRODUCTION

CRC³ is the fourth most common cancer and ranks third as a cause of cancer-related mortality in the United States (1). CRCs are intrinsically resistant to anticancer drugs. CRCs produce excess PGs, particularly PGE₂, of which the synthesis is catalyzed by *COX* enzymes from arachidonic acid (2). Isoforms of the *COX* gene include constitutively expressed *COX-1* and inducible *COX-2* (2). *COX-2* is an intermediate response gene that encodes a *M_r* 71,000 cytoplasmic protein that is up-regulated at sites of inflammation (3), and in human colorectal (4, 5) and several other epithelial malignancies (6–10). Deletion of the murine *COX-2* gene in ^{APCΔ716}mice dramatically reduced intestinal polyp number (11). Furthermore, overexpression of the human *COX-2* gene in mammary glands of transgenic mice resulted in mammary tumors, indicating that *COX-2* was sufficient to induce tumorigenesis (12).

Epidemiological data indicate that regular and prolonged intake of

NSAIDs are associated with a 40–50% reduction in CRC incidence (13–16). Treatment of familial adenomatous polyposis patients with the NSAID sulindac or the selective *COX-2* inhibitor celecoxib have been shown to effectively regress existing colorectal adenomas relative to placebo (17, 18). Selective *COX-2* inhibitors were developed given that inhibition of *COX-1* is largely responsible for the adverse effects associated with NSAID treatment (19, 20). NSAIDs are also potent chemopreventive agents in animal models of colon cancer (11, 21–23). In intestinal tissues from animals and humans treated with NSAIDs, modulation of apoptotic rates was found in association with tumor inhibition/regression, suggesting a mechanism for their chemopreventive effects (24–28). Studies by ourselves (6) and others (29–31) have shown that sulindac sulfide, a nonselective *COX* inhibitor, and NS398, a selective *COX-2* inhibitor, can inhibit growth and induce apoptosis in cultured colon and pancreatic cancer cell lines. However, the molecular and biochemical pathways responsible for the proapoptotic effects of NSAIDs remain poorly understood.

Inhibition of *COX* and PG synthesis by NSAIDs may not explain many of the experimental results obtained. Evidence for *COX*-independent effects include the observation that NSAIDs can inhibit the growth of colon and pancreatic cancer cells devoid of *COX-2* expression as they do for those producing *COX-2* (6, 31, 32). Furthermore, NSAID metabolites lacking the ability to inhibit *COX* enzymes, i.e., sulindac sulfone, can induce apoptosis *in vitro* (30) and inhibit experimental colon cancer (33). In some (34), but not other (35) reports, the addition of PGE₂ to NSAID-treated colon cancer cells reversed their growth inhibitory and proapoptotic effects. Moreover, murine embryonic fibroblast cells with homologous knockout of *COX-1* and *COX-2* alleles remain sensitive to the antiproliferative and proapoptotic effects of NSAIDs (36).

Anticancer drugs eliminate tumor cells by inducing their apoptosis (37). Therefore, modulation of apoptotic susceptibility is a critical determinant of therapeutic efficacy. Apoptosis is controlled by two major pathways including a mitochondrial (38) and a membrane DR pathway (39). Mitochondrial release of cytochrome *c* into the cytosol has been shown in cell-free systems to be rate limiting for the activation of caspases and endonucleases (40, 41). Cytosolic cytochrome *c* activates procaspase-9 by binding to Apaf1 in the presence of dATP, leading to caspase-9 activation and subsequent activation of downstream effector caspases, including caspase-3, with triggering of apoptosis (42). Activated caspases perform proteolytic cleavage events involved in apoptosis (38). The antiapoptotic Bcl-2 or Bcl-X_L proteins act on mitochondria to inhibit cytochrome *c* release and to prevent opening of the megachannel (38, 43, 44). Accordingly, Bcl-2 proteins can prevent caspase activation, Bax redistribution to the mitochondria, and apoptosis after exposure to anticancer drugs (41, 43–45). Caspase-8 is a proximal caspase that plays a critical role in the DR-mediated apoptotic pathway, used by Fas, tumor necrosis factor α, and tumor necrosis factor-related apoptosis-inducing ligand, that is independent of cytochrome *c* release (39). Membrane DRs are activated by their respective ligands and engage adaptor molecules and caspases, including proximal caspase-8 (39, 46).

In this report, we determined whether forced *COX-2* expression can modulate susceptibility to apoptosis induced by NSAIDs and 5-FU.

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³ The abbreviations used are: CRC, colorectal cancer; PG, prostaglandin; *COX*, cyclooxygenase; NSAID, non-steroidal anti-inflammatory drug; DR, death receptor; 5-FU, 5-fluorouracil; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcription-PCR.

After 4 decades, 5-FU, an inhibitor of DNA synthesis, remains the most active and widely used single agent in the treatment of CRC. We examined whether these drugs use the mitochondrial apoptotic pathway by determining the effects of forced COX-2 expression on cytochrome *c* release, caspase-9, -3, and -8 activation, and Bcl-2 expression.

MATERIALS AND METHODS

Cell Culture and Drug Treatment. The HCT-15 human colon cancer cell line was obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere including 5% CO₂. Cells were seeded at a density of 3×10^6 cells/100-mm dishes 24 h before drug treatment. The effect of NS398 (Cayman Chemical, Ann Arbor, MI), sulindac sulfide (Cell Pathways Inc., Horsham, PA) and 5-FU (Acros Organics, Fairlawn, NJ) on apoptosis were studied. Drugs were dissolved in 100% DMSO and then diluted in medium for experiments. The final concentration of DMSO was maintained at 0.1%. After 24 h, fresh medium was added containing NS398, sulindac sulfide, or 5-FU, and cells were incubated for 48 h. Both floating and attached cells were harvested for analysis of apoptosis using TUNEL.

COX-2 cDNA Transfection. Cells (3×10^5 in 2 ml of RPMI 1640) were plated in six-well Costar tissue culture plates. Twenty-four h later, cells were transfected with 2.5 μ g pSG5-COX2 plasmid, which contains a full-length COX-2 cDNA in the pSG expression vector and 0.5 μ g pcDNA1, which contains neomycin-resistant marker, as described previously (47). Transfection was performed with LipofectAMINE reagent (Life Technologies, Inc.) according to vendor's instruction. Positive transfectants were selected in RPMI 1640 containing 500 μ g/ml geneticin (Gibco Life Technologies, Inc., Carlsbad, CA). Cell lines were established from individual colonies using cloning cylinders.

PGE₂ Synthesis. Medium from 2-day serum-depleted 35-mm cultures were pooled and [¹⁴C] arachidonic acid (0.10 μ Ci/ml media) was added. The dishes were refed with the radioactive medium and incubated for 16 h, as described previously (48). PGE₂ analysis was performed by Dr. Susan Fischer (M. D. Anderson Cancer Center).

TUNEL Assay. DNA strand breaks, consistent with apoptosis, were detected by the TUNEL assay, which was performed using the In Situ Cell Death Detection kit (Roche Diagnostics, Mannheim, Germany). Cultured cells were treated for 48 h with NS398 (40–160 μ M), sulindac sulfide (40–160 μ M), or 5-FU (0.04–0.16 mM). Cells were then harvested and fixed using 4% paraformaldehyde in PBS (pH 7.4) for 1 h at room temperature. Cells were resuspended in 100 μ l of 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice for permeabilization and then labeled with fluorescein dUTP and stained with propidium iodide, according to the manufacturer's instructions. Thereafter, the cells were analyzed for apoptosis using a FACScan flow cytometer (Epics Profile; Coulter Corp., Hialeah, FL).

MTT Assay. Cells were seeded in 96-well plates at a density of 2×10^4 and allowed to attach overnight. The cells were then treated with NS398 (200 μ M) for 48 h. Medium was then replaced with 100 μ l of MTT solution (Sigma Chemical Co., St. Louis, MO) at a final concentration of 1 mg/ml/well. After incubation at 37°C for 4 h, the supernatant was aspirated, and 100 μ l of 10% SDS was added to each well and incubated for 8 h at 37°C. Absorbance was measured at a wavelength of 75 nm using a microplate reader.

Immunoblotting. Cells were washed with cold PBS and suspended in a lysis buffer [20 mM HEPES (pH 7.5), 100 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EGTA (pH 7.5), 1 μ g/ml aprotinin, 1 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride] on ice for 15 min. After removal of cell debris by centrifugation, the protein concentration in cell lysates was determined using a Bradford protein assay (Bio-Rad, Richmond, CA). Samples containing 50 μ g of protein were added to SDS-PAGE loading buffer with 5% β -mercaptoethanol, and loaded in a 10% (COX-2), 12% (Bcl-2), or 15% (cytochrome *c*) polyacrylamide gels. Proteins were then transferred to nitrocellulose membranes. After blocking the blot with 5% milk in PBS containing 0.1% Tween 20, the membrane was incubated for 1 h at room temperature with an anti-COX-2 (1:1000; Cayman Chemicals), anti- β -actin (1:3000; Sigma Chemical Co.), anti-Bcl-2 (1:80; Dako Corporation, Denmark), or anticytochrome *c* (1:1000; PharMingen, San Diego, CA) monoclonal antibodies. Blots were then

incubated with secondary antibodies conjugated with peroxidase (Bio-Rad) for 1 h at room temperature. The signal was detected by chemiluminescence using the ECL detection kit (Amersham, Arlington Heights, IL).

RT-PCR. Total cellular RNA was extracted from HCT15 \pm COX-2 cells using the RNeasy Mini kit (Qiagen, Valencia, CA), per the manufacturer's instructions. The RNA was quantified by determining absorbance at 260 nm. After reverse transcription of 10 μ g RNA, the cDNA product was amplified by PCR using a gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA) in a 20 μ l final reaction volume for 38 cycles using One Step RT-PCR kit (Qiagen, Hilden, Germany). Amplification of the *Bcl-2* and reference gene β -actin were performed in one reaction. The following primer pairs were used: *Bcl-2*: sense, 5'-CTTTGGAAGGATGGCGCACGCTGG-3'; antisense, 5'-AGGCACTTCACTTGTGGCTCAGAGG-3'; size: 723 bp; β -actin sense, 5'-GTTGCTATCCAGGCTGTGC-3'; antisense, 5'-CGTACGGCTGTCC-TACG-3'; size: 541 bp. The cycling conditions were as follows: 30 min at 50°C, 15 min at 95°C, followed by 38 cycles at 94°C for 30 s; 61°C for 1 min, and 72°C for 1 min. The amplified products were separated on 1.5% agarose gels stained with 0.1 μ g/ml of ethidium bromide and photographed under UV light.

Analysis of Cytochrome *c* Release. Floating and attached cells were collected after incubation with sulindac sulfide (160 μ M), NS398 (200 μ M), or 5-FU (0.16 mM) for 48 h. Cells were washed once in PBS (pH 7.5) and then added to Buffer A [0.25 M sucrose, 1.0 mM DTT, 2.0 mM HEPES (pH 7.5), 1.0 mM KCl, 1.5 mM MgCl₂, 1.0 mM sodium-EDTA, 1.0 mM sodium-EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 1 μ g/ml leupeptin, 1 μ g/ml pepstatin, and 1 μ g/ml aprotinin]. Cells were collected, and then homogenized and centrifuged at $1,000 \times g$ for 15 min at 4°C. The supernatants were centrifuged again at $20,000 \times g$ for 45 min at 4°C. The supernatant (cytosolic fraction) was collected, and protein concentration was determined by the Bradford protein assay (Bio-Rad). To determine cytochrome *c* release into the cytosol, immunoblotting was performed using anticytochrome *c* monoclonal antibody (PharMingen).

Caspase Activation Assays. Cells were treated with NS398 (200 μ M), sulindac sulfide (160 μ M), or 5-FU (0.16 mM) for 48 h, then harvested and lysed. The protein concentration in the cell lysates was determined, and 200 μ g of protein was added to 50 μ l of lysis buffer for each assay. Caspase-9 activity was determined using the caspase-9/Mch6 Colorimetric Protease Assay kit (MBL, Nagoya, Japan) using LEHD-NA as a substrate with incubation at 37°C for 2 h. Samples were then read at a wavelength of 405 nm. For caspase-3, cells were treated as described above, and 50 μ g protein from cell lysates was analyzed for caspase-3 activity using the Caspase-3 Cellular Activity Assay kit (Calbiochem, San Diego, CA) using Ac-DEVD-NA as a substrate and a reaction time of 2 h at 37°C. Samples were read as described above. A specific

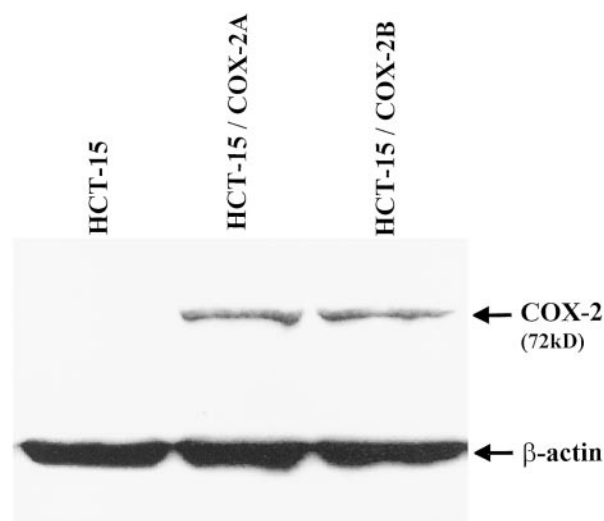


Fig. 1. Immunoblot analysis of COX-2 expression in the HCT-15 colon cancer cell line stably transfected with the COX-2 cDNA. HCT-15 cells were transfected with the neomycin-resistance gene alone or cotransfected with the COX-2 expression vector. Two G418 resistant clones (HCT-15/COX-2A and HCT-15/COX-2B) overexpressing COX-2 proteins were isolated.

Fig. 2. Forced COX-2 expression attenuates apoptosis induction by NSAIDs and 5-FU. Cultured HCT-15 (○) and COX-2 transfected HCT-15/COX-2-A (●), and HCT-15/COX-2-B (■) cells were incubated for 48 h with sulindac sulfide (A), NS398 (B), or 5-FU (C). Cells were then harvested, and apoptosis was determined using the TUNEL assay. Induction of apoptosis by all three of the drugs was dosage-dependent. Forced COX-2 expression significantly attenuated drug induced apoptosis compared with parental HCT-15 cells. Data points represent the mean triplicate of value determinations; bars, \pm SD.

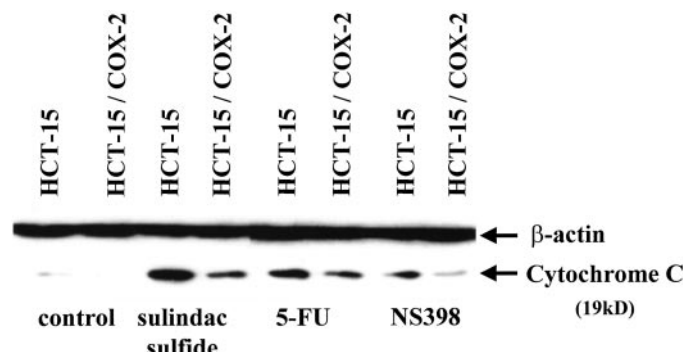
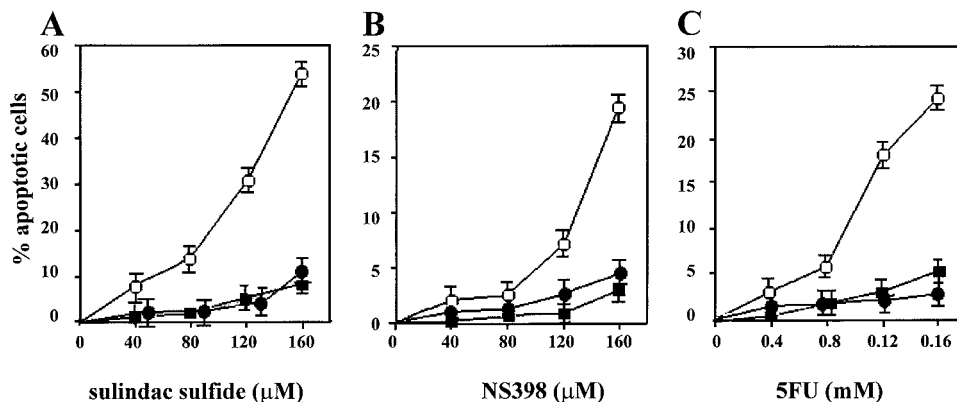


Fig. 3. Cytosolic cytochrome *c* protein expression is induced by treatment with sulindac sulfide, NS398, and 5-FU. Forced COX-2 expression in HCT-15/COX-2A cells was found to attenuate cytochrome *c* release induced by sulindac sulfide (160 μ M), NS398 (200 μ M), or 5-FU (0.16 mM) compared with parental HCT-15 cells. Cells were incubated with drugs for 48 h, and both floating and attached cells were collected for immunoblot analysis.

inhibitor of caspase-3 Ac-DQMD-CHO (Biomol Research Labs, Plymouth Meeting, PA) was used in separate experiments. Caspase-8 activity was determined with the Caspase-8 Assay kit (Calbiochem) using Ac-IETD-pNA as a substrate with incubation at 37°C for 2 h.

RESULTS

The HCT-15 colon cancer cell line that lacks endogenous COX-2 expression and PG production (32) was stably transfected with the

human COX-2 cDNA. Two G418-resistant clones expressing a high level of COX-2 were selected for subsequent experiments (Fig. 1). COX-2 transfectants had elevated PGE₂ levels relative to parental cells. The effect of forced COX-2 expression on the apoptotic susceptibility of these cells to sulindac sulfide, NS398, and 5-FU was determined. As shown in Fig. 2, A–C, all three of the drugs induced apoptosis in a dosage-dependent manner. NS398 was less potent as an inducer of apoptosis on an equimolar basis relative to sulindac sulfide (6). Overexpression of COX-2 in both clones (2A and 2B) significantly attenuated apoptosis induction by all three of the drugs relative to parental HCT-15 cells. Analysis of PGE₂ levels after drug treatment revealed suppression by NSAIDs and no change for 5-FU (data not shown).

To determine the mechanism of COX-2-mediated resistance to apoptosis induction, we analyzed key steps in the mitochondrial pathway of apoptosis. Cytosolic cytochrome *c* protein expression was determined by immunoblotting in cytosolic extracts of drug-treated and untreated HCT-15 \pm COX-2 cells. Incubation of cells with sulindac sulfide, NS398, or 5-FU were found to induce cytochrome *c* expression (Fig. 3). Forced COX-2 expression inhibited cytochrome *c* release compared with parental HCT-15 cells (Fig. 3). Given that Bcl-2 can inhibit mitochondrial cytochrome *c* release (41, 43–45), we hypothesized that COX-2 transfectants may overexpress Bcl-2 with resultant inhibition of cytochrome *c* release and attenuation of drug-induced apoptosis. To address this issue, we analyzed Bcl-2 expression in COX-2 transfected cells. Interestingly, we found that both

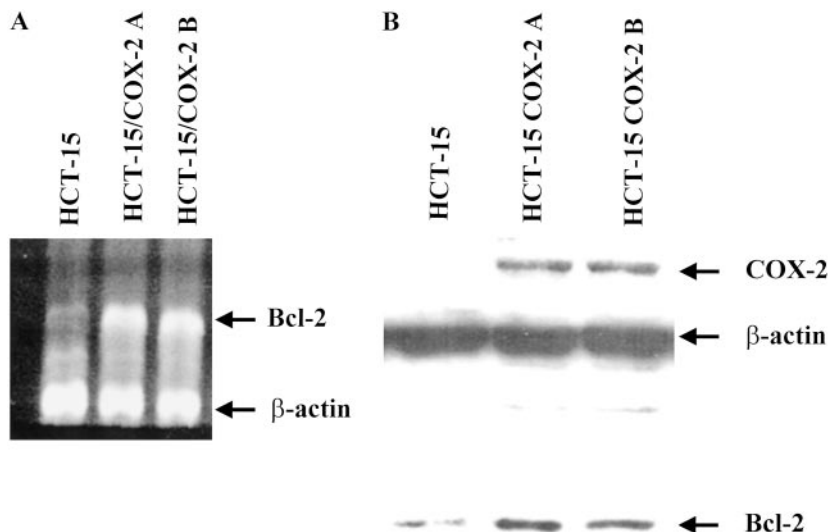


Fig. 4. COX-2 stable transfection induces up-regulation of Bcl-2 mRNA and protein in HCT-15/COX-2 cells relative to parental cells. A, RT-PCR analysis of Bcl-2 in two HCT-15/COX-2 clones relative to parental HCT-15 cells. B, immunoblot analysis demonstrates increased Bcl-2 expression in two clones overexpressing COX-2 proteins relative to low level Bcl-2 in parental HCT-15 cells.

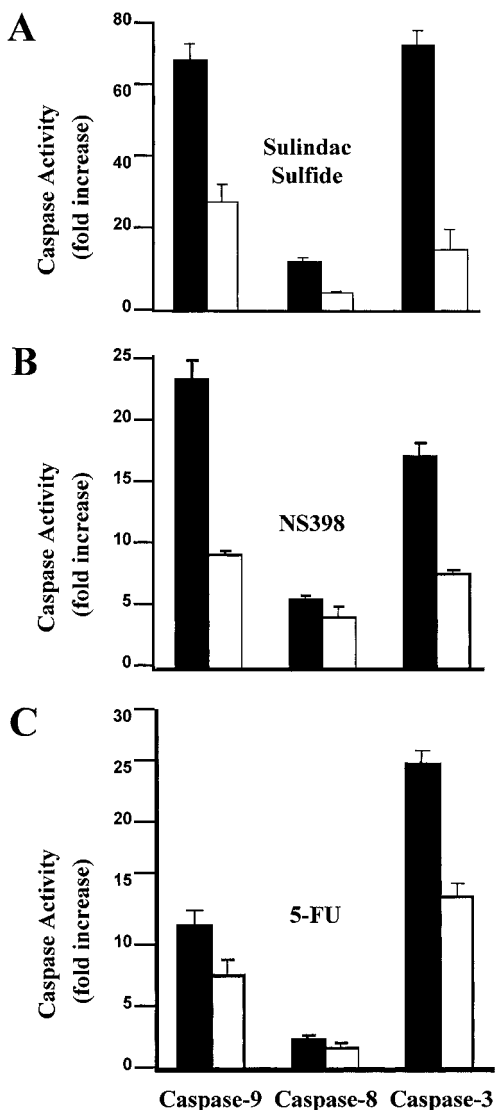


Fig. 5. Forced COX-2 expression attenuates caspase activation by drug treatment in HCT-15 cells. Cells were treated with sulindac sulfide (160 μ M), NS398 (200 μ M), or 5-FU (0.16 mM) for 48 h, and floating and attached cells were collected. Cell lysates were analyzed for caspase-9, -8, and -3 activation as described in "Materials and Methods." All three drugs induced activation of caspase-9 and -3, and to a lesser extent, caspase-8. HCT-15/COX-2A cells (□) showed significantly reduced caspase-9 and -3 activation by these drugs relative to parental HCT-15 cells (■). Caspase-8 activation was significantly reduced in HCT-15/COX-2A cells treated with sulindac sulfide, but not after NS398 or 5-FU treatment. Data represent mean of triplicate measurements; bars, \pm SD.

Bcl-2 mRNA and protein levels were increased in untreated COX-2 overexpressing cells relative to parental cells (Fig. 4, A and B). The increase in Bcl-2 in COX-2 transfectants relative to parental cells was maintained during drug treatment. These data indicate that COX-2 overexpression can up-regulate Bcl-2.

Events downstream of cytochrome *c* include caspase-9 and -3 activation. Caspase-9 is an initiator caspase that is triggered by cytochrome *c* and Apaf1 binding, and when activated, cleaves and activates caspase-3 (42). Treatment of cells with sulindac sulfide, NS398, or 5-FU were found to significantly increase caspase-9 and caspase-3 activities (Figs. 5, A–C). In COX-2 overexpressing cells, a significant reduction in drug-induced caspase-9 and -3 activation was observed relative to parental HCT-15 cells. The reduction in caspase-9 activity by COX-2 varied from 1.5- to 2.5-fold after treatment with these three drugs. A 1.8–4.2-fold reduction in caspase-3 was detected in COX-2 transfectants after drug treatment. To verify the role of caspase-3 as a

downstream effector of apoptosis in HCT-15 cells, we used the specific caspase-3 inhibitor Ac-DQMD-CHO (2 nM). After treatment with NS398 (200 μ M) for 48 h this inhibitor was shown to restore cell survival, using the MTT assay, in COX-2 transfected and parental cells (data not shown). We also analyzed caspase-8 activity given that it plays a critical role in DR-mediated apoptosis that is independent of cytochrome *c* (39). Caspase-8 activity was increased by drug treatment but to a lesser extent than were caspase-9 and -3 (Fig. 5, A–C). Forced COX-2 expression attenuated caspase-8 activation induced by sulindac sulfide but not by NS398 or 5-FU.

To determine whether COX-2-mediated inhibition of caspase-3 activation by 5-FU was because of PGE₂ overproduction, we added NS398 (40 and 80 μ M). NS398 treatment failed to reverse the COX-2-mediated suppression of caspase-3 activation by 5-FU, indicating that this effect, and presumably resultant apoptosis, are likely to be independent of COX-2 enzymatic activity (Fig. 6). This result is consistent with experiments shown in Fig. 2, A–C, wherein apoptosis resistance was observed using doses of NSAIDs that have been shown to inhibit COX-2 enzymatic activity and PG production (2).

DISCUSSION

Evidence indicates that induction of tumor cell apoptosis by NSAIDs and 5-FU is an important mechanism of their antitumor effects (24–33). Conflicting data exist in different cell lines as to whether the level of COX-2 can predict the proapoptotic effects of NSAIDs *in vitro* (6, 31, 49). Therefore, we generated HCT-15 colon cancer cells ectopically overexpressing COX-2 to determine whether COX-2 can modulate their apoptotic susceptibility to NSAIDs and 5-FU. We found that apoptosis induction by sulindac sulfide, NS398, and 5-FU was significantly attenuated in COX-2 transfectants compared with parental cells. Additionally, NS398 failed to reverse COX-2-mediated suppression of caspase-3 activation by 5-FU. Inhibition of apoptosis by COX-2 appears to be independent of COX-2 enzymatic activity as shown by the inability of NSAIDs, which suppressed PGE₂, to reverse COX-2-mediated inhibition of apoptosis. Furthermore, Hanif *et al.* (32) found that treatment of HCT-15 cells with PGE₂ failed to reverse apoptosis induction by sulindac sulfide. Forced

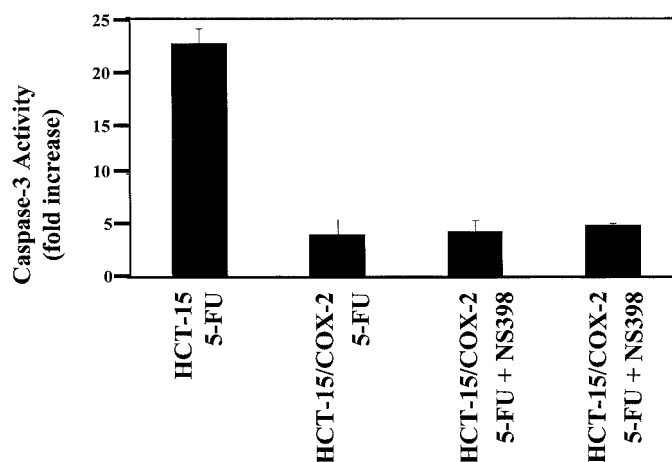


Fig. 6. Forced COX-2 expression attenuates 5-FU-induced activation of caspase-3 that is not reversed by the addition of NS398. We determined whether NS398 could reverse COX-2-mediated inhibition of caspase-3 activation. HCT-15 and HCT-15/COX-2A cells were treated with 5-FU (0.16 mM) alone or combined with NS398 (40, 80 μ M) for 48 h. Cell lysates were analyzed for caspase-3 activation. HCT-15/COX-2A cells showed attenuated caspase-3 activation by 5-FU treatment relative to parental cells. Addition of NS398 failed to restore caspase-3 activation suggesting that COX-2-mediated suppression of caspase-3 activity is independent of COX-2 enzymatic activity. Data represent mean of triplicate measurements; bars, \pm SD.

COX-2 expression has also been shown to confer resistance to butyrate-induced apoptosis in nontransformed intestinal epithelial cells (50). These findings indicate that COX-2 influences the malignant phenotype, as shown in studies where COX-2 altered cell adhesion and increased metastatic potential (50). Evidence suggests that COX-2 expression may contribute to *in vivo* drug resistance in colorectal neoplasms. Epithelial COX-2 expression was found to be significantly reduced at baseline in colorectal adenomas from a limited number of familial adenomatous polyposis patients with complete adenoma regression on sulindac compared with nonresponders (51).

Sulindac sulfide, NS398, and 5-FU engaged the mitochondrial apoptotic pathway involving cytosolic cytochrome *c* release and subsequent activation of upstream caspase-9 and downstream caspase-3. Importantly, COX-2 overexpression attenuated these effects resulting in an inhibition of apoptosis. Induction of the mitochondrial apoptotic pathway by NSAIDs has also been shown in SW620 and other colon cancer cell lines (52, 53). We found that NSAIDs and 5-FU also induced caspase-8 activation but to a much lesser extent than for caspase-9 and -3. Recently, sulindac sulfide was shown to increase expression of membrane DR5 in colon and prostate cancer cells, and to activate caspase-8 (54). Furthermore, we found that COX-2 overexpression inhibited sulindac sulfide-induced caspase-8 activation. In a separate study, we found that DR5, but not DR4, was transcriptionally repressed in COX-2 overexpressing HCT-15 cells, indicating that COX-2 can modulate the level of DR5, and thereby regulate the membrane DR pathway (55). This finding provides a potential mechanism for the observed reduction in caspase-8 activation by sulindac sulfide in COX-2 transfected cells. Taken together, we provide evidence for predominant involvement of the mitochondrial pathway but also the caspase-8-dependent apoptotic pathway in NSAID and 5-FU-induced apoptosis in human colon cancer cells.

Evidence indicates that Bcl-2 acts to stabilize mitochondrial membrane integrity by preventing cytochrome *c* release, and subsequent caspase activation and apoptosis (40, 41, 43, 44). These effects contribute to the ability of Bcl-2 to inhibit apoptosis-induction by a wide variety of cytotoxic drugs (37). To determine whether attenuated cytochrome *c* release in drug-treated COX-2 transfectants was related to alterations in Bcl-2, we analyzed Bcl-2 expression. COX-2 transfectants, in the absence or presence of drug treatment, showed up-regulation of Bcl-2 mRNA and protein levels relative to parental cells. Therefore, increased Bcl-2 may contribute to attenuated cytochrome *c* release, reduced caspase-9 and -3 activation, and resultant inhibition of apoptosis in COX-2 overexpressing colon cancer cells. Our results are consistent with the finding that forced COX-2 expression in nontransformed rat intestinal epithelial cells was associated with Bcl-2 induction and resistance to butyrate-induced apoptosis (50). In transgenic mice, forced COX-2 expression in mammary glands was associated with the development of mammary tumors and induction of Bcl-2 expression (12). Addition of PGE₂ to HCA-7 colon cancer cells that constitutively express COX-2 was shown to inhibit apoptosis induced by celecoxib (SC-58125) and to induce Bcl-2 expression (56). Maintenance of elevated Bcl-2 levels in cells treated with NSAIDs, as shown in this report, suggests that up-regulation of Bcl-2 can occur independently of PGE₂. Whereas reduced apoptotic susceptibility mediated by COX-2 is attributable, in part, to up-regulation of Bcl-2 and its inhibition of cytochrome *c* release, the molecular mechanism underlying COX-2-mediated up-regulation of Bcl-2 is unknown at this time. The Bcl-2 promoter is transcriptionally regulated by wild-type *p53* via a *p53*-dependent response element (57); however, HCT-15 cells express mutant *p53* proteins (58). In contrast to Bcl-2, Bax and Bid are believed to promote apoptosis by increasing cytochrome *c* release (38). Zhang *et al.* (53) found that disruption of the Bax gene in human colon cancer cells abrogated cytochrome *c*-

dependent apoptosis induction by NSAIDs. These data indicate that NSAIDs engage the mitochondrial apoptotic pathway in colon cancer cells that is regulated by Bcl-2 and Bax (53).

In conclusion, our data demonstrate that COX-2 can confer resistance to apoptosis induction by diverse antineoplastic agents in transformed cells and suggest that COX-2 overexpression may represent a novel mechanism of intrinsic drug resistance in human CRCs. NSAIDs and 5-FU engaged the cytochrome *c*-dependent apoptotic pathway that was inhibited by forced COX-2 expression. Up-regulation of Bcl-2 by COX-2 provides a potential mechanism for reduced apoptotic susceptibility. Given that COX-2 is overexpressed in the majority of human CRCs, our findings have important implications for the prevention and treatment of this disease.

REFERENCES

- Greenlee, R., Hill-Harmon, M. B., Murray, T., and Thun, M. J. Cancer statistics, 2001. *CA Cancer J. Clin.*, 51: 15–36, 2001.
- Williams, C. S., Mann, M., and DuBois, R. N. The role of cyclooxygenases in inflammation, cancer, and development. *Oncogene*, 18: 7908–7915, 1999.
- Jones, D. A., Carlton, D. P., McIntyre, T. M., Zimmerman, G. A., and Prescott, S. M. Molecular cloning of human prostaglandin endoperoxidase synthase type 2 and demonstration of expression in response to cytokines. *J. Biol. Chem.*, 268: 9049–9054, 1993.
- Eberhart, C. E., Coffey, R. J., Radhika, A., Giardiello, F. M., Ferrebach, S., and Dubois, R. N. Up-regulation of cyclooxygenase2 gene expression in human colorectal adenomas and adenocarcinomas. *Gastroenterology*, 107: 1183–1188, 1994.
- Sinicrope, F. A., Lemoine, M., Xi, L., Lynch, P. M., Cleary, K. R., Shen, Y., and Frazier, M. L. Reduced expression of cyclooxygenase 2 proteins in hereditary nonpolyposis colorectal cancers relative to sporadic cancers. *Gastroenterology*, 117: 350–358, 1999.
- Molina, M. A., Sitja-Arnau, M., Lemoine, M. G., Frazier, M. L., and Sinicrope, F. A. Increased cyclooxygenase-2 expression in human pancreatic carcinomas and cell lines: Growth inhibition by nonsteroidal anti-inflammatory drugs. *Cancer Res.*, 59: 4356–4362, 1999.
- Wilson, K. T., Fu, S., Ramanujam, K. S., Meltzer, S. J. Increased expression of inducible nitric oxide synthase and cyclooxygenase-2 in Barrett's esophagus and associated adenocarcinomas. *Cancer Res.*, 58: 2929–2934, 1998.
- Ristimaki, A., Honkanen, N., Jankala, H., Sipponen, P., and Harkonen, M. Expression of cyclooxygenase-2 in human gastric carcinoma. *Cancer Res.*, 57: 1276–1280, 1997.
- Half, E., Tang, X. M., Gwyn, K., Sahin, A., Wathen, K., and Sinicrope, F. A. Cyclooxygenase-2 expression in human breast cancers and adjacent ductal carcinoma *in situ*. *Cancer Res.*, 62: 1676–1681, 2002.
- Hida, T., Yatabe, Y., Achiwa, H., Muramatsu, H., Kozaki, K., Nakamura, S., Ogawa, M., Mitsudomi, T., Sogiura, T., and Takahashi, T. Increased expression of cyclooxygenase-2 occurs frequently in human lung cancers, specifically in adenocarcinomas. *Cancer Res.*, 58: 3761–3764, 1998.
- Oshima, M., Dinchuk, J. E., Kargman, S. L., Oshima, H., Hancock, B., Kwong, E., Trzaskos, J. M., Evans, J. F., and Taketo, M. M. Suppression of intestinal polyposis in Apc $\delta 716$ knockout mice by inhibition of cyclooxygenase 2 (COX-2). *Cell*, 87: 803–809, 1996.
- Liu, C. H., Chang, S., Narko, K., Trifan, O. C., Wu, M., Smith, E., Haudenschild, C., Lane, T. F., and Hla, T. Overexpression of cyclooxygenase-2 is sufficient to induce tumorigenesis in transgenic mice. *J. Biol. Chem.*, 276: 18563–18569, 2001.
- Thun, M. J., Namboodiri, M. M., and Heath, C. W., Jr. Aspirin use and reduced risk of fatal colon cancer. *N. Engl. J. Med.*, 325: 1593–1596, 1991.
- Giovannucci, E., Rimm, E. B., Stampfer, M. J., Colditz, G. A., Ascherio, A., and Willett, W. C. Aspirin use and the risk for colorectal cancer and adenoma in male health professionals. *Ann. Intern. Med.*, 121: 241–246, 1994.
- Giovannucci, E., Egan, K. M., Hunter, D. J., Stampfer, M. J., Colditz, G. A., Willett W. C., and Speizer, F. E. Aspirin and the risk of colorectal cancer in women. *N. Eng. J. Med.*, 333: 609–614, 1995.
- Smalley, W., Ray, W. A., Daugherty, J., and Griffin, M. R. Use of nonsteroidal anti-inflammatory drugs and incidence of colorectal cancer: a population-based study. *Arch. Intern. Med.*, 159: 161–166, 1999.
- Giardiello, F. M., Hamilton, S. R., Krush, A. J., Piantadosi, S., Hylind, L. M., Celano, P., Booker, S. V., Robinson, C. R., and Offerhaus, G. J. Treatment of colonic and rectal adenomas with sulindac in familial adenomatous polyposis. *N. Eng. J. Med.*, 328: 1313–1316, 1993.
- Steinbach, G., Lynch, P., Phillips, R., Wallace, M., Hawk, E., Gordon, G., Wakabayashi, N., Saunders, B., Shen, Y., Fujimura, T., Su, L., and Levin, B. The effect of celecoxib, a cyclooxygenase-2 inhibitor, in familial adenomatous polyposis. *N. Eng. J. Med.*, 342: 1946–1952, 2000.
- Feldman, M., and McMahon, A. T. Do cyclooxygenase-2 inhibitors provide benefits similar to those of traditional nonsteroidal anti-inflammatory drugs, with less gastrointestinal toxicity? *Ann. Intern. Med.*, 132: 134–143, 2000.
- Bombardier, C., Laine, L., Reicin, A., Shapiro, D., Burgos-Vargas, R., Davis, B., Day, R., Ferraz, M. B., Hawkey, C. J., Hochberg, M. C., Kvien, T. K., and Schnitzer, T. J. VIGOR Study Group. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. VIGOR Study Group. *N. Engl. J. Med.*, 343: 1520–1528, 2000.

21. Kawamori, T., Rao, C. V., Seibert, K., and Reddy, B. S. Chemopreventive activity of celecoxib, a specific cyclooxygenase-2 inhibitor, against colon carcinogenesis. *Cancer Res.*, 58: 409–412, 1998.
22. Jacoby, R. F., Marshall, D. J., Newton, M. A., Novakovic, K., Tutsch, K., Cole, C. E., Lubet, R. A., Kelloff, G. J., Verma, A., Moser, A. R., and Dove, W. F. Chemoprevention of spontaneous intestinal adenomas in the Apc Min mouse model by the nonsteroidal anti-inflammatory drug piroxicam. *Cancer Res.*, 56: 710–714, 1996.
23. Boolbol, S. K., Dannenberg, A. J., Chadburn, A., Martucci, C., Guo, X. J., Ramonetti, J. T., Abreu-Goriss, M., Newmark, H. L., Lipkin, M. L., DeCosse, J. J., and Bertagnoli, M. M. Cyclooxygenase-2 overexpression and tumor formation are blocked by sulindac in a murine model of familial adenomatous polyposis. *Cancer Res.*, 56: 2556–2560, 1996.
24. Mahmoud, N. N., Boolbol, S. K., Dannenberg, A. J., Mestre, J. R., Bilinski, R. T., Martucci, C., Newmark, H. L., Chadburn, A., and Bertagnoli, M. M. The sulfide metabolite of sulindac prevents tumors and restores enterocyte apoptosis in a murine model of familial adenomatous polyposis. *Carcinogenesis (Lond.)*, 19: 87–91, 1998.
25. Keller, J. J., Offerhaus, G. J., Polak, M., Goodman, S. N., Zahurak, M. L., Hyland, L. M., Hamilton, S. R., and Giardiello, F. M. Rectal epithelial apoptosis in familial adenomatous polyposis patients treated with sulindac. *Gut*, 45: 822–828, 1999.
26. Samaha, H. S., Kelloff, G. J., Stelle, V., Rao, C. V., and Reddy, B. S. Modulation of apoptosis by sulindac, curcumin, phenylethyl-3-methylcaffeate, and 6-phenylhexyl isothiocyanate: apoptotic index as a biomarker in colon cancer chemoprevention and promotion. *Cancer Res.*, 57: 1301–1305, 1997.
27. Roy, H. K., Karoski, W. J., Ratashak, A., and Smyrk, T. C. Chemoprevention of intestinal tumorigenesis by nabumetone: induction of apoptosis and Bcl-2 downregulation. *Br. J. Cancer*, 84: 1412–1416, 2001.
28. Li, H., Schut, H. A., Conran, P., Kramer, P. M., Lubet, R. A., Steele, V. E., Hawk, E. E., Kelloff, G. J., and Pereira, M. A. Prevention by aspirin and its combination with α -difluoromethylornithine of azoxymethane-induced tumors, aberrant crypt foci and prostaglandin E2 levels in rat colon. *Carcinogenesis (Lond.)*, 20: 425–430, 1999.
29. Shiff, S. J., Qiao, L., Tsai, L. L., and Rigas, B. Sulindac sulfide, an aspirin-like compound, inhibits proliferation, causes cell cycle quiescence, and induces apoptosis in HT-29 colon adenocarcinoma cells. *J. Clin. Investig.*, 96: 491–503, 1995.
30. Piazza, G. A., Rahm, A. L., Krutzsch, M., Sperl, G., Paranka, N. S., Gross, P. H., Brendel, K., Burt, R. W., Alberts, D. S., and Pamukcu, R. Antineoplastic drugs sulindac sulfide and sulfone inhibit cell growth by inducing apoptosis. *Cancer Res.*, 55: 3110–3116, 1995.
31. Elder, D. J., Halton, D. E., Hague, A., and Paraskeva, C. Induction of apoptotic cell death in human colorectal carcinoma cell lines by a cyclooxygenase-2 (COX-2)-selective nonsteroidal anti-inflammatory drug: independence from COX-2 protein expression. *Clin. Cancer Res.*, 3: 1679–1683, 1997.
32. Hanif, R., Pittas, A., Feng, Y., Koutsos, M. I., Qiao, L., Staiano-Coico, L., Shiff, S. I., and Rigas, B. Effects of nonsteroidal anti-inflammatory drugs on proliferation and on induction of apoptosis in colon cancer cells by a prostaglandin-independent pathway. *Biochem. Pharmacol.*, 52: 237–245, 1996.
33. Piazza, G. A., Alberts, D. S., Hixson, L. J., Paranka, N. S., Li, H., Finn, T., Bogert, C., Guillen, J. M., Brendel, K., Gross, P. H., Sperl, G., Ritchie, J., Burt, R. W., Ellsworth, L., Ahnen, D. J., and Pamukcu, R. Sulindac sulfone inhibits azoxymethane-induced colon carcinogenesis in rats without reducing prostaglandin levels. *Cancer Res.*, 57: 2909–2915, 1997.
34. Tsujii, M., and DuBois, R. N. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. *Cell*, 83: 493–501, 1995.
35. Elder, D. J., Halton, D. E., Crew, T. E., and Paraskeva, C. Apoptosis induction and cyclooxygenase-2 regulation in human colorectal adenoma and carcinoma cell lines by the cyclooxygenase-2-selective non-steroidal anti-inflammatory drug NS-398. *Int. J. Cancer*, 86: 553–560, 2000.
36. Zhang, X., Morham, S. G., Lagenbach, R., and Young, D. A. Malignant transformation and antineoplastic actions of nonsteroidal anti-inflammatory drugs (NSAIDs) on cyclooxygenase-null embryo fibroblasts. *J. Exp. Med.*, 190: 451–460, 1999.
37. Reed, J. C., Miyashita, T., Takayama, S., Wang, H. G., Sato, T., Krajewski, S., Aime-Sempe, C., Bodrug, S., Kitada, S., and Hanada, M. BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J. Cell. Biochem.*, 60: 23–32, 1996.
38. Green, D. R., and Reed, J. C. Mitochondria and apoptosis. *Science (Wash. DC)*, 281: 1309–1312, 1998.
39. Ashkenazi, A., and Dixit, V. M. Apoptosis control by death and decoy receptors. *Curr. Opin. Cell Biol.*, 11: 225–260, 1999.
40. Martinou, J. C., Desagher, S., and Antonsson, B. Cytochrome c release from mitochondria: all or nothing. *Nat. Cell Biol.*, 2: E41–E43, 2000.
41. Kluck, R. M., Bossy-Wetzell, E., Green, D. R., and Newmeyer, D. D. The release of cytochrome c from mitochondria: A primary site for Bcl-2 regulation of apoptosis. *Science (Wash. DC)*, 275: 1132–1136, 1997.
42. Li, P., Nijhawan, D., Budihardjo, I., Srinivasula, S. M., Ahmad, M., Alnemri, E. S., and Wang, X. Cytochrome c and dATP-dependent formation of Apaf-2/Caspase-9 complex initiates an apoptotic protease cascade. *Cell*, 91: 479–489, 1997.
43. Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., and Wang, X. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science (Wash. DC)*, 275: 1129–1132, 1997.
44. Tsujimoto, Y., and Shimizu, S. Bcl-2 family: life-or-death switch. *FEBS Lett.*, 466: 6–10, 2000.
45. Murphy, K. M., Ranganathan, V., Farnsworth, M. L., Kavallaris, M., and Lock, R. B. Bcl-2 inhibits Bax translocation from cytosol to mitochondria during drug-induced apoptosis of human tumor cells. *Cell Death Differ.*, 7: 102–111, 2000.
46. Sheridan, J. P., Marsters, S. A., Pitti, R. M., Gurney, A., Skubatch, M., Baldwin, D., Ramakrishnan, L., Gray, C. L., Baker, K., Wood, W. I., Goddard, A. D., Godowski, P., and Ashkenazi, A. Control of TRAIL-induced apoptosis by a family of signaling and decoy receptors. *Science (Wash. DC)*, 277: 818–821, 1997.
47. Tatebe, S., Unate, H., Sinicrope, F. A., Sakatani, T., Sugamura, K., Makino, M., Ito, H., Savaraj, N., Kaibara, N., and Kuo, M. T. Expression of heavy subunit of γ -glutamylcysteine synthetase (γ -GCSH) in human colorectal carcinoma. *Int. J. Cancer*, 97: 21–27, 2002.
48. Maldiv, R. E., Kim, Y., Muga, S. J., and Fischer, S. M. Prostaglandin E2 regulation of cyclooxygenase expression in keratinocytes is mediated via cyclic nucleotide-linked prostaglandin receptors. *J. Lipid Res.*, 41: 873–881, 2000.
49. Sheng, H., Shao, J., Kirkland, S. C., Isakson, P., Coffey, R. J., Morrow, J., Beauchamp, R. D., and DuBois, R. N. Inhibition of human colon cancer cell growth by selective inhibition of cyclooxygenase-2. *J. Clin. Investig.*, 99: 2254–2259, 1997.
50. Tsujii, M., Kawano, S., and DuBois, R. N. Cyclooxygenase-2 expression in human colon cancer cells increases metastatic potential. *Proc. Natl. Acad. Sci. USA*, 94: 3336–3340, 1997.
51. Keller, J. J., Offerhaus, G. J., Drillenburg, P., Caspers, E., Musler, A., Ristamaki, A., and Giardiello, F. M. Molecular analysis of sulindac-resistant adenomas in familial adenomatous polyposis. *Clin. Cancer Res.*, 7: 4000–4007, 2001.
52. Li, M., Wu, X., and Xu, X.-C. Induction of apoptosis in colon cancer cells by cyclooxygenase-2 inhibitor NS398 through a cytochrome c-dependent pathway. *Clin. Cancer Res.*, 7: 1010–1016, 2001.
53. Zhang, L., Yu, J., Park, B. H., Kinzler, K. W., and Vogelstein, B. Role of BAX in the apoptotic response to anticancer agents. *Science (Wash. DC)*, 290: 989–992, 2000.
54. Huang, Y., He, Q., Hillman, M. J., Rong, R., and Sheikh, M. S. Sulindac sulfide-induced apoptosis involves death receptor 5 and the caspase 8-dependent pathway in human colon and prostate cancer cells. *Can. Res.*, 61: 6918–6924, 2001.
55. Tang, X., Sun, Y., Half, E., Kuo, M., and Sinicrope, F. A. Cyclooxygenase-2 overexpression inhibits death receptor 5 expression and confers resistance to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis in human colon cancer cells. *Cancer Res.*, 62: 4903–4908, 2002.
56. Sheng, H., Shao, J., Morrow, J. D., Beauchamp, R. D., and DuBois, R. N. Modulation of apoptosis and Bcl-2 expression by prostaglandin E2 in human colon cancer cells. *Cancer Res.*, 58: 362–366, 1998.
57. Miyashita, T., Harigai, M., Hanada, M., and Reed, J. C. Identification of a p53-dependent negative response element in the bcl-2 gene. *Cancer Res.*, 54: 3131–3135, 1994.
58. Chinery, R., Brockman, J. A., Peeler, M. O., Shyr, Y., Beauchamp, R. D., and Coffey, R. J. Antioxidants enhance the cytotoxicity of chemotherapeutic agents in colorectal cancer: a p53-independent induction of p21^{WAF1/CIP1} via C/EBP β . *Nat. Med.*, 3: 1233–1241, 1997.