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Advances in Brief

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[1]

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Abstract

The inducible *cyclooxygenase-2* (*COX-2*) gene regulates prostaglandin biosynthesis, is up-regulated in colorectal cancers, and can influence apoptotic susceptibility. We determined whether forced COX-2 expression modulates apoptosis induction by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of tumor necrosis factor ligand family, and examined determinants of the apoptotic pathway, including membrane death receptors (DR-4 and DR-5). HCT-15 colon cancer cells lacking endogenous COX-2 proteins were stably transfected with the *COX-2* cDNA and incubated with TRAIL. Forced COX-2 expression significantly attenuated TRAIL-induced apoptosis and was associated with transcriptional repression of DR-5 and up-regulation of Bcl-2. COX-2 transfectants showed reduced DR-5 mRNA and protein expression as well as reduced caspase-8, caspase-3, and caspase-9 activation relative to parental cells. Sulindac sulfide treatment restored DR-5 expression and, when combined with TRAIL, reduced cell viability to a greater extent than did either drug alone. In summary, modulation of DR-5 and Bcl-2 levels by COX-2 attenuates TRAIL-induced apoptosis and represents a novel mechanism of intrinsic drug resistance in human colon cancer cells.

Introduction

COX[3] enzymes catalyze the conversion of arachidonic acid to prostaglandins and related eicosanoids (1). Overexpression of COX-2, but not COX-1, is associated with high levels of PGE_2 in colorectal tumors relative to normal mucosa (2). Two COX isoforms have been identified and include constitutive COX-1 and inducible COX-2 (1). *COX-2* is an intermediate response gene that is induced by cytokines, growth factors, and tumor promoters and is up-regulated at sites of inflammation and in certain neoplastic tissues, including colorectal neoplasms (1–3). The importance of COX-2 in intestinal tumorigenesis was shown by mating COX-2 knockout mice to $APC^{\Delta 716}$ mice, which resulted in a dramatic reduction in intestinal polyp burden in double knockouts (4). Forced COX-2 expression has been shown to increase cellular adhesion and to confer resistance to butyrate-induced apoptosis in rat intestinal epithelial cells (5). Furthermore, COX-2 overexpression can reduce antineoplastic drug and Fas-mediated apoptosis in human colon and cholangiocarcinoma cells, respectively (6, 7). To date, the mechanisms by which COX-2 can inhibit apoptosis are poorly understood. Apoptosis is controlled via two major pathways, including one that originates at the cell membrane and another that involves the mitochondria (8, 9). The membrane DR pathway involves DRs such as Fas, TNF-R1, DR-3, DR-4, and DR-5, that are activated by their respective ligands and engage the intracellular apoptotic machinery (9, 10). TRAIL (also known as Apo2L) is a newly identified member of the TNF ligand family that can induce a rapid caspase-dependent apoptosis in transformed cells but not in most normal cells (11, 12). Accordingly, TRAIL is being evaluated in human studies as a potential anticancer agent. TRAIL activates DR-4 (DR-4/TRAIL-R1) and DR-5 (DR-5/TRAIL-R2), whereas TNF-α, Fas ligand, and Apo3L are ligands for TNF-R1, Fas, and DR-3, respectively (10). Activation of DR-4 and DR-5 results in the recruitment of the intracellular adaptor molecule Fas-associated death domain that engages proximal caspase-8 at the receptor site (13). Loss of caspase-8 activation can contribute to resistance to TRAIL-induced apoptosis (14). Furthermore, the combination of TRAIL and certain anticancer drugs induced apoptosis to a greater extent than did either drug alone (15).

Most anticancer drugs induce apoptosis by engaging the cytochrome *c*-dependent, mitochondrial apoptotic pathway (8). This pathway is negatively regulated by the antiapoptotic Bcl-2 protein through suppression of cytochrome *c* release (8, 16). We (6) and others (17) have shown that NSAIDs engage the mitochondrial pathway and our data indicate that COX-2 overexpression attenuates apoptosis induction by NSAIDs and 5-fluorouracil, and is associated with Bcl-2 up-regulation. NSAIDs inhibit COX enzymes and can induce apoptosis and inhibit cell proliferation in cultured colorectal cancer cell lines (18, 19) and in intestinal epithelia from animal models of colon cancer (20) and in humans (21), suggesting that modulation of these processes contributes to their antitumor effects *in vivo.* NSAIDs are effective chemopreventive agents against experimental colon cancer and in patients with familial adenomatous polyposis were shown to reduce the number and size of colorectal polyps relative to placebo (reviewed in Ref. 22). Furthermore, epidemiological studies have shown a 40–50% reduction in the incidence of colorectal cancer in subjects taking NSAIDs for extended periods of time (reviewed in Ref. 22). In this report, we determined the effect of ectopic expression of *COX-2* upon TRAIL-mediated apoptosis in HCT-15 colon cancer cells and examined the apoptotic pathway involved in such modulation. Specifically, we determined whether forced COX-2 expression alters DR-4 and DR-5 levels and/or caspase activation by TRAIL. The effect of NSAIDs on DR-4 and DR-5 levels and the effect of the combination of NSAIDs and TRAIL upon cell death were also examined.

Materials and Methods

Cell Culture and Drug Treatment. The HCT-15 and SW480 human colon cancer cell lines were obtained from American Type Culture Collection (Manassas, VA) and were grown in RPMI 1640 or Leibovitz's L-15 medium, respectively, supplemented with 8% fetal bovine serum and antibiotics with 10

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[3] The abbreviations used are: COX, cyclooxygenase; DR, death receptor; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; NSAID, nonsteroidal anti-inflammatory drug; PGE_2, prostaglandin E_2; RT-PCR, reverse transcription-PCR; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

mM HEPES. Cells were grown in a monolayer and maintained at 37°C in a humidified atmosphere including 5% CO_2. Cells were seeded at a density of 3×10^6 cells/100-mm dish for 24 h before drug treatment. The effect of TRAIL (Calbiochem, San Diego, CA), NS398 (Cayman Chemical, Ann Arbor, MI), and sulindac sulfide (Cell Pathways, Inc., Horsham, PA) on cell viability were studied. Drugs were dissolved in 100% DMSO and then diluted in media for experiments. The final concentration of DMSO was maintained at 0.1%. After 24 h, fresh medium containing drug was added, and cells were then incubated for 48 h. Both floating and attached cells were harvested for subsequent analysis.

Transfection of COX-2 cDNA. Cells (3×10^5 in 2 ml of RPMI 1640) were plated in 6-well Costar tissue culture plates. Twenty-four h later, cells were transfected with vector alone or with 2.5 μg of pSG5-COX-2 plasmid, which contains a full-length COX-2 cDNA in the pSG expression vector (a gift of Dr. Robert Kulmacz, University of Texas Medical School, Houston, TX) and 0.5 mg of pcDNA1, which contains a neomycin-resistant marker, as described previously (23). A similar procedure was previously followed for stable transfection of SW480 cells with the Bcl-2 cDNA (PC3-Bcl-2; gift of Dr. Timothy McDonnell, University of Texas M. D. Anderson Cancer Center). Transfection was performed with Lipofectamine reagent (Life Technologies, Inc.) according to the vendor's instructions. Positive transfectants were selected in RPMI 1640 containing 500 μg/ml geneticin (Life Technologies, Inc.). Cell lines were established from individual colonies using cloning cylinders.

cDNA Preparation and Quantitative Multiplex PCR. Total RNA was extracted and purified from cultured cells using the RNeasy Mini kit (Qiagen, Valencia, CA), per the manufacturer's instructions. The RNA was quantified by determining absorbance at 260 nm. One μg of total RNA from each sample was reverse transcribed into cDNA using Superscript II reverse transcriptase (Life Technologies, Inc.) in a volume of 20 μl. The cDNA product was diluted to 100 μl and then amplified by PCR using a Gene Amp PCR System 2700 (Applied Biosystems, Foster City, CA) with β-actin as an internal control. To produce an optimal PCR amplification, a linear correlation between PCR cycle numbers and products was established. Ten μl of PCR reaction mixture contained 1 μl of diluted cDNA sample, 1 unit of Hotstart polymerase (Qiagen, Valencia, CA), 0.1 mM deoxynucleotide triphosphates, 1 μl of DMSO, 50 ng of primers of DR-4 (forward, 5-CGA TGT GGT CAG AGC TGG TAC AGC-3; reverse, 5-GGA CAC GGC AGA GCC TGT GCC ATC-3′), DR-5 (forward, 5-AAG ACC CTT GTG CTC GTT G-3; reverse, 5-TCA CCT GAA TCA CAC CTG G-3), and β-actin (forward, 5′-GTT GCT ATC CAG GCT GTG C-3′; reverse, 5′-GCA TCC TGT CGG CAA TGC-3′). The reaction was performed using the following program: 94°C for 15 min to activate the polymerase, 30 cycles at 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, and then an additional extension step of 72°C for 7 min. The PCR products were electrophoresed on a 2.5% agarose gel and stained with ethidium bromide, photographed under UV light, and then quantified by scanning densitometry relative to the absorbance of β-actin.

Western Blotting. Cells were washed with cold PBS and suspended in a 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 placed on ice for 60 min and then centrifuged at 14,000 rpm for 30 min to remove cellular debris. The protein concentration in the samples was measured using a Bradford protein assay kit (Bio-Rad, Richmond, CA). Samples containing 50 μg of protein were added to a SDS-PAGE loading buffer and then loaded in a 12% denaturing polyacrylamide gel. Proteins were then transferred electrophoretically to Hybond-C nitrocellulose membranes (Amersham-Pharmacia Biotech, Inc., Piscataway, NJ). Membranes were blocked using 5% nonfat dry milk in 10 mM PBS for 1 h at room temperature and then incubated for 2 h at room temperature with monoclonal antibodies against DR-4, DR-5 (1:500; ProSci, Inc., Poway, CA), COX-2 (1:1000; Cayman Chemical, Ann Arbor, MI), Bcl-2 (1:100; Dako, Copenhagen, Denmark), or cytochrome *c* (1:800; PharMingen, San Diego, CA). Anti-β-actin (1:5000; Sigma, St Louis, MO) was used as an internal control for protein loading. Blots were washed three times with PBS containing 0.1% Tween 20 for 15 min each and incubated with a second antibody supplied with the ECL kit (Amersham-Pharmacia) for 1 h at room temperature. The signal was detected by chemiluminescence using the ECL detection kit. Scanning densitometry was conducted for protein quantification.

Cytochrome *c* Release from Mitochondria. Untreated and TRAIL-treated HCT-15 cells transfected with either vector alone or the COX-2 expression vector were gently homogenized in an ice-cold buffer containing 0.1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, 2 mM HEPE-KOH (pH 7.5), 1 mM KCl, 1.5 mM $MgCl_2$, 1 mM sodium-EDTA, 1 mM sodium EGTA, 0.25 M sucrose, proteinase inhibitor (1 μg/ml leupeptin, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). The homogenate was centrifuged at 900 $\times$ *g* for 10 min at 2°C, and the supernatant was centrifuged at 8000 $\times$ *g* for 30 min at 2°C. The resulting supernatant solution was centrifuged at 100,000 $\times$ *g* for 30 min at 2°C to remove any mitochondrial contamination. The protein concentration in the cytosolic fraction was determined, and the fraction was denatured in Laemmli buffer (250 mM Tris-HCl, 4% SDS, 10% glycerol, 0.006% bromophenol blue, and 2% β-mercaptoethanol, pH 6.8) at 100°C. Immunoblotting was performed as described above using a 15% polyacrylamide gel.

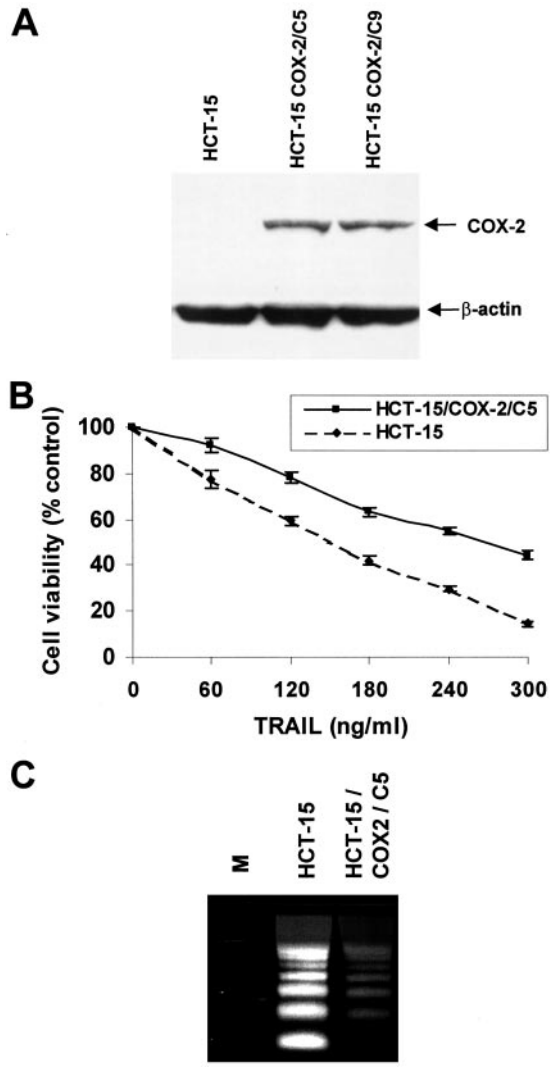

Fig. 1. *A*, immunoblot of COX-2 expression in HCT-15 colon cancer cells stably transfected with vector alone or with the *COX-2* cDNA. Two G418-resistant clones (C5 and C9) overexpressing COX-2 (M_r 72,000) proteins are shown. *B*, dose-dependent reduction in cell viability by TRAIL in HCT-15 ± COX-2 cells. Forced COX-2 expression significantly attenuated the reduction in cell viability induced by TRAIL relative to parental cells (MTT assay). Data represent means of triplicate determinations; *bars*, SE. *C*, oligonucleosomal DNA fragmentation assay demonstrates that TRAIL (200 ng/ml; 24 h) is inducing DNA strand breaks consistent with apoptosis in HCT-15 ± COX-2 cells.

Northern Blot. Five μg of total RNA extracted from cultured cells were electrophoresed on 1% formaldehyde-agarose gel and transferred to a nylon membrane (Scheicher & Schuell, Keene, NH). The cDNA for DR-5 was synthesized and ^{32}P-labeled ([α-^{32}P]dCTP, 3,000 Ci/mmol; ICN, Costa Mesa, CA) using PCR primers (same as described for RT-PCR). Blots were hybridized in Rapid-hyb buffer (Amersham-Pharmacia) overnight at 65°C. Blots were then autoradiographed, stripped, and reprobed with β-actin to normalize for differences in loading and transfer.

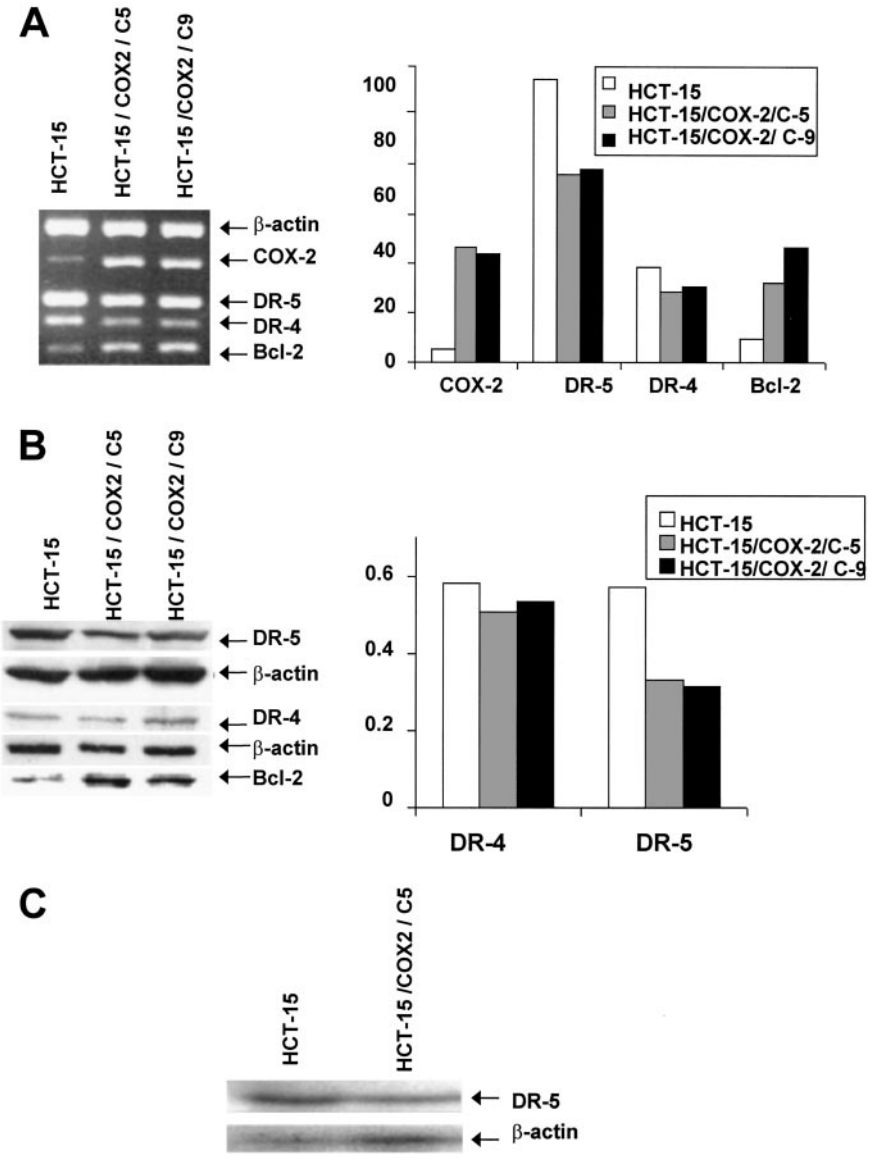

Fig. 2. *A*, analysis of DR-4, DR-5, and COX-2 mRNA expression in HCT-15 and HCT-15/COX-2 (C5 and C9) cells by RT-PCR with quantitation by scanning densitometry. DR-4 and to a greater extent DR-5 mRNA levels are reduced in COX-2 transfectants (C5 and C9) relative to parental cells ($P < 0.001$). *B*, immunoblotting demonstrates a reduction in DR-5, but not DR-4, protein expression in COX-2-overexpressing cells, as shown quantitatively by densitometry. *C*, Northern blotting demonstrates transcriptional repression of DR-5 mRNA in COX-2 transfectants relative to parental cells.

MTT Assay. For analysis of cell viability after TRAIL treatment, 4×10^3 cells of each cell line were seeded in triplicate in 96-well plates in 0.1 ml of medium for 24 h. Medium was then replaced with media containing TRAIL (30–300 ng/ml) and incubated for an additional 24 h. Incubation of cells with sulindac sulfide (20 μM) or NS398 (50 μM) for 24 h followed by TRAIL (0–40 ng/ml) for 24 h was also performed. Medium was then removed, and 100 μl of fresh medium containing 10 μl of MTT (Sigma; 5 mg/ml in PBS) were added. Cells were incubated at 37°C for 3 h, medium was aspirated, and the cells were washed with PBS. Then, 0.15 ml of DMSO was added to each well, and absorbance at a wavelength of 540 nm was measured using a Spectra Count microplate reader (Perkin Elmer Life Sciences, Boston, MA). Cell survival was calculated: $(\%) = 100 \times A_{treatment}/A_{control}$.

Caspase Activation Assays. Cells (2×10^7) in 10-cm dishes were treated with TRAIL (30 ng/ml) for 24 h and then harvested by scraping in lysis buffer (Calbiochem). The cell lysates were placed on ice for 10 min and centrifuged at 10,000 $\times$ *g* for 10 min. The protein concentration in supernatants was determined. 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. For caspase-3, cells were treated as described above, and caspase-3 activity was determined using the Caspase-3 Cellular Activity Assay kit (Calbiochem) using Ac-DEVD-NA as a substrate and a reaction time of 2 h at 37°C. A specific inhibitor of caspase-3, Ac-DQMD-CHO (Biomol Research Labs, Plymouth Meeting, PA), was used. 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. For all caspase activation assays, 100 μg of extracted protein were mixed with the reaction buffer and substrate (included in kits) in 96-well plates and incubated for 5 h at 37°C. Samples were then read at a wavelength of 405 nm, and absorbances at 3 h of reaction were recorded. Caspase activity was evaluated by the absolute absorbance of TRAIL-treated cells subtracted from the absolute absorbance of untreated control cells.

DNA Fragmentation. After treatment with 200 ng/ml of TRAIL for 24 h, both floating and attached cells were harvested. Cells were pelleted by centrifugation and resuspended in Tris-EDTA buffer (pH 8.0). Cells were 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 the soluble (fragmented) from the 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 then stained with ethidium bromide. Gels were photographed under UV light.

Results and Discussion

The HCT-15 colon cancer cell line that lacks endogenous COX-2 protein expression was stably transfected with the human *COX-2*

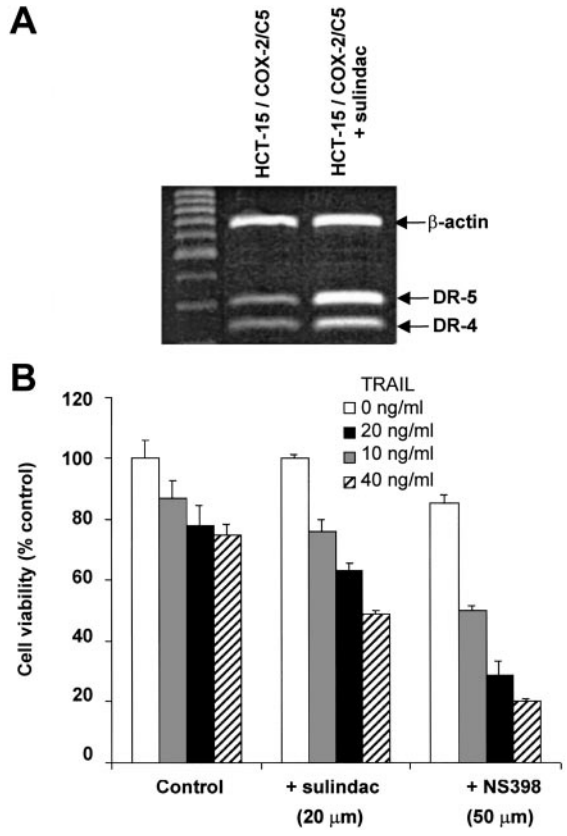

Fig. 3. *A*, DR-4 and DR-5 mRNA expression are induced by treatment of COX-2-transfected HCT-15 (C5) cells with sulindac sulfide (20 μm; 24 h), as determined by RT-PCR. *B*, C5 cells were incubated with TRAIL (0–40 ng/ml) alone and in combination with sulindac sulfide (20 μM) or NS398 (50 μM). The combination of TRAIL and sulindac sulfide or NS398 reduced cell viability to a greater extent than did TRAIL treatment alone. Mean values are shown for triplicate experiments; *bars*, SE.

cDNA. Two G418-resistant clones (C5 and C9) expressing a high level of COX-2 proteins were selected for subsequent experiments (Fig. 1*A*). COX-2 transfectants had elevated PGE_2 levels relative to parental cells (data not shown). The effect of forced COX-2 expression on cell viability after TRAIL treatment was determined. As shown in Fig. 1*B*, the ligand TRAIL reduced cell viability in HCT-15 ± COX-2 cells in a dosage-dependent manner. Overexpression of COX-2 was found to significantly attenuate this effect. At 120 ng/ml of TRAIL, COX-2 transfectants showed a 20% reduction in cell viability *versus* a 40% reduction observed for parental cells (Fig. 1*B*). To determine whether the reduction in cell viability induced by TRAIL was attributable to apoptosis, a DNA fragmentation assay was performed oligonucleosomal. DNA laddering was seen in TRAIL-treated HCT-15 ± COX-2 cells consistent with apoptosis (Fig. 1*C*); COX-2 overexpression appeared to reduce DNA fragmentation by TRAIL. Suppression of COX-2 enzymatic activity by NSAIDs induces apoptosis, whereas ectopic COX-2 expression, as shown here, can inhibit apoptosis. Consistent with our findings are data in cholangiocarcinoma cells where increased COX-2 or PGE_2 were shown to confer resistance to Fas-mediated apoptosis, and this effect was reversed by the selective COX-2 inhibitor, NS-398 (7). To determine the mechanism of COX-2-mediated resistance to TRAIL-induced apoptosis, we analyzed membrane DR expression levels and caspase activation. DR-4 and DR-5 mRNA and protein expression were determined in two clones (C5 and C9) overexpressing COX-2, using RT-PCR and immunoblotting, respectively, with quantification by scanning densitometry. HCT-15 cells expressed DR-4 and DR-5, and both clones overexpressing COX-2 showed reduced DR-5 and to a lesser extent DR-4, mRNA (Fig. 2*A*). DR-5 protein expression was also reduced (Fig. 2*B*). Furthermore, Northern blotting demonstrated that COX-2 transcriptionally repressed DR-5 expression (Fig. 2*C*). We then determined whether the NSAID sulindac sulfide can modulate DR-4 and DR-5 expression levels. COX-2-overexpressing cells were treated with sulindac sulfide (20 μM for 24 h), and DR-4 and DR-5 mRNA levels were analyzed by RT-PCR. We found that sulindac sulfide increased both DR-4 and DR-5 mRNA levels in these cells (Fig. 3*A*). Similarly, Huang *et al.* (24) found that sulindac sulfide treatment increased DR-5 expression in human colon cancer cells. Induction of DR-5 in HCT-15 cells is p53 independent because these cells contain mutant p53 proteins. DR-5 induction has been shown to occur in both p53-positive and p53-negative colon cancer cell lines (24). These data suggest that increased DR levels by sulindac sulfide may contribute to the proapoptotic effect of this drug. In this regard, certain anticancer drugs have been shown to induce apoptosis through induction of DRs (25). Activation of DR-5 using an agonistic DR-5 antibody (TRA-8) has been shown to increase TRAIL-induced apoptosis *in vitro* and *in vivo*, indicating that modulation of the level of DR-5 regulates the apoptotic response to TRAIL (26). The finding of increased DR-5 expression by sulindac sulfide suggests that inhibition of COX-2 enzymatic activity by NSAIDs can reverse COX-2-mediated down-regulation of DR-5. To test this hypothesis, we determined

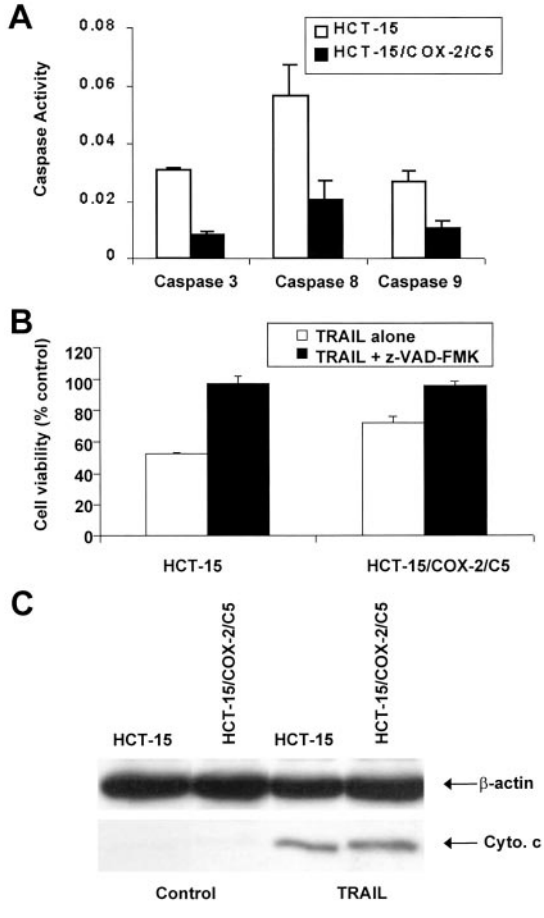

Fig. 4. *A*, forced COX-2 expression attenuates TRAIL-mediated activation of caspase-3, caspase-8, and caspase-9. COX-2 transfectants (C5) and parental HCT-15 cells were incubated with TRAIL (30 ng/ml for 24 h). Caspase activation was determined in cell lysates by colometric assay (see "Materials and Methods"). Activities of all three caspases were significantly inhibited in C-5 cells ($P < 0.001$). Results of triplicate experiments are shown; *bars*, SE. *B*, a pan-caspase inhibitor z-Val-Ala-Asp-fluoromethyl ketone, was found to restore cell survival after TRAIL (30 ng/ml) treatment in both C5 and parental HCT-15 cells. *C*, TRAIL (200 ng/ml; 4 h) induced cytochrome *c* (*Cyto. c*) release to an equivalent extent in C5 and HCT-15 cells.

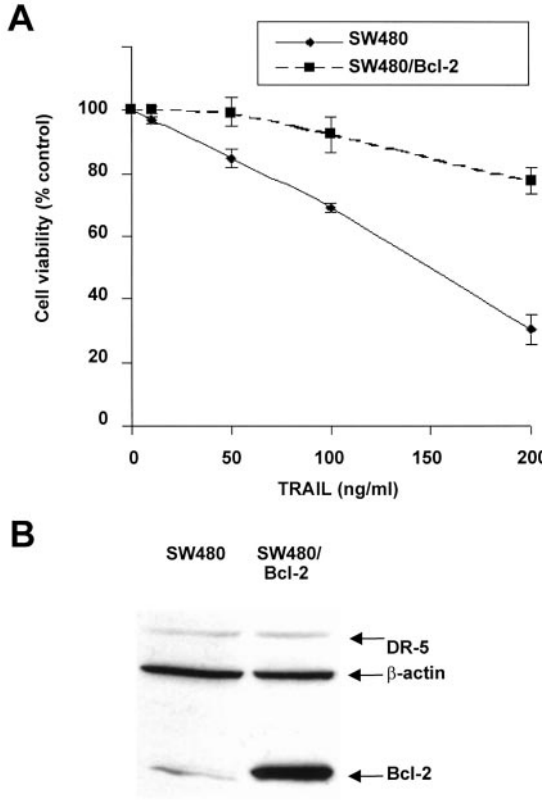

Fig. 5. *A*, forced Bcl-2 expression in SW480 colon cancer cells confers resistance to TRAIL-induced apoptosis. Parental SW480 and Bcl-2 stable transfectants were incubated with TRAIL (0–200 ng/ml) for 24 h. TRAIL treatment reduced cell viability, measured by the MTT assay, in a dosage-dependent manner. Bcl-2-overexpressing cells showed protection from TRAIL-induced cell death relative to vector-only transfectants. *B*, DR-5 protein expression was found to be similar in Bcl-2 transfectants and in parental SW480 cells, indicating that modulation of cell survival by Bcl-2 is independent of alterations in the level of DR-5.

the effect of the combination of sulindac sulfide and TRAIL treatment upon cell viability. We found that the combination reduced cell viability to a greater extent than did either drug alone (Fig. 3*B*), indicating that inhibition of COX-2 enhances apoptotic susceptibility to TRAIL.

TRAIL treatment of HCT-15 cells was found to increase caspase-8 and caspase-3, as well as caspase-9, activation (Fig. 4*A*). Caspase-9 activation by TRAIL was also found in PC-3 prostate cancer cells, suggesting that multiple caspase pathways are involved in TRAIL-induced cell death (27). Forced COX-2 expression significantly attenuated TRAIL-induced caspase-8, caspase-3, and caspase-9 activation relative to parental HCT-15 cells (Fig. 4*A*). An ~30% reduction in caspase activation was found in COX-2 transfectants after drug treatment. Furthermore, the pan-caspase inhibitor, z-Val-Ala-Asp-fluoromethyl ketone, was shown to restore cell viability after TRAIL treatment (Fig. 4*B*). Thus, COX-2 attenuates TRAIL-mediated apoptosis by inhibiting caspase activation and DR expression. Given that caspase-9 is triggered by cytochrome *c* and Apaf1 binding (28), we analyzed cytochrome *c* release by TRAIL. TRAIL treatment induced cytochrome *c* expression in COX-2-overexpressing cells and in parental cells to an equivalent extent (Fig. 4*C*). Induction of cytochrome *c* by TRAIL is likely attributable to caspase-8-related cleavage of BID, which has been reported to translocate to mitochondria and to induce cytochrome *c* release (29). Bcl-2 expression was then analyzed, given its known role as an inhibitor of cytochrome *c* release and subsequent apoptosis (16). COX-2 transfectants showed up-regulation of Bcl-2 mRNA and protein (Fig. 2, *A* and *B*). This finding is consistent with results in rat intestinal epithelial cells where ectopic COX-2 expression was associated with up-regulation of Bcl-2 (30). Furthermore, exogenous PGE_2 was shown to induce Bcl-2 expression and to inhibit apoptosis induced by a selective COX-2 inhibitor in colon cancer cells (30). In our study, COX-2-transfected cells overexpressing Bcl-2 did not show attenuated cytochrome *c* induction by TRAIL at 4 h (Fig. 4*C*). Similarly, Walczak *et al.* (14) and Keogh *et al.* (31) found that Bcl-2 failed to block cytochrome *c* release after exposure to TRAIL in Jurkat cells. To determine whether Bcl-2 can block TRAIL-mediated cell death, we used SW480 colon cancer cells stably transfected with the Bcl-2 cDNA. We found that the Bcl-2-overexpressing cells were protected from TRAIL; however, TRAIL was still able to kill cells overexpressing Bcl-2 (Fig. 5*A*). Partial protection from TRAIL-mediated cell death is consistent with data in PC-3 prostate cancer cells (27) and in non-small cell lung cancer cells (32). In contrast, Bcl-2 failed to protect 8226 and ARP-1 myeloma cells from TRAIL-induced apoptosis (33). Interestingly, Bcl-2 was shown to block activation of caspase-8, a component of the DR complex, by the chemopreventive agent curcumin (34). To exclude an effect of Bcl-2 on the level of DR-5, we examined DR-5 expression in Bcl-2-overexpressing SW480 cells. DR-5 protein expression was not altered by forced Bcl-2 expression (Fig. 5*B*), indicating that COX-2-mediated down-regulation of DR-5 is independent of Bcl-2.

In summary, we demonstrate for the first time that forced COX-2 expression significantly attenuates TRAIL-induced apoptosis in human colon cancer cells. COX-2 overexpression transcriptionally repressed DR-5 expression and was associated with attenuated caspase activation and up-regulation of Bcl-2. COX-2 inhibition by sulindac sulfide restored DR-5 expression and augmented TRAIL-mediated cell death. The additive effect of sulindac sulfide and TRAIL upon cell death suggests that pharmacological inhibition of COX-2 may be useful in augmenting TRAIL-mediated apoptosis and has implications for the prevention and treatment of human colorectal cancer.

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