Cyclooxygenase-2 Inhibition Sensitizes Human Colon Carcinoma Cells to TRAIL-Induced Apoptosis through Clustering of DR5 and Concentrating Death-Inducing Signaling Complex Components into Ceramide-Enriched Caveolae

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Abstract

Cyclooxygenase-2 (COX-2) is up-regulated in human colon carcinomas, and its inhibition is associated with a reduction in tumorigenesis and a promotion of apoptosis. However, the mechanisms responsible for the antitumor effects of COX-2 inhibitors and how COX-2 modulates apoptotic signaling have not been clearly defined. We have shown that COX-2 inhibition sensitizes human colon carcinoma cells to tumor necrosis factor–related apoptosis-inducing ligand (TRAIL)–induced apoptosis by inducing clustering of the TRAIL receptor DR5 at the cell surface and the redistribution of the death-inducing signaling complex components (DR5, FADD, and procaspase-8) into cholesterol-rich and ceramide-rich domains known as caveolae. This process requires the accumulation of arachidonic acid and sequential activation of acid sphingomyelinase for the generation of ceramide within the plasma membrane outer leaflet. The current study highlights a novel mechanism to circumvent colorectal carcinoma cell resistance to TRAIL-mediated apoptosis using COX-2 inhibitors to manipulate the lipid metabolism within the plasma membrane. (Cancer Res 2005; 65(24): 11447-58)

Introduction

During the 1990s, an association was made between regular consumption of nonsteroidal anti-inflammatory drugs (NSAID) and a reduction in the incidence of colon cancer (1). Initial clinical trials showed that the NSAID sulindac sulfide reduced the number and size of colorectal adenomas in patients with familial adenomatous polyposis (FAP) by a mechanism postulated to involve the inhibition of cyclooxygenase-2 (COX-2), the inducible isoenzyme of the COX family of rate-limiting enzymes required for the synthesis of eicosanoids, such as prostaglandins. Transcription of the COX-2 gene is rapidly induced in response to cytokines, growth factors, and numerous pharmacologic agents and in pathologic conditions (2, 3). Further to the finding that COX-2 expression is commonly up-regulated in various human cancers, including colon carcinomas, genetic and pharmacologic studies have shown a cause-and-effect connection between expression of COX-2 and carcinogenesis (3–7). Studies in vitro or in animal models suggest that prostaglandins produced by COX-2 reduce the rate of apoptosis induction and increase proliferation of cancer cells (1). Furthermore, in human subjects with FAP treated with specific COX-2 inhibitors Celecoxib (8) or Rofecoxib (9), regression of colorectal polyps was shown. Although specific COX-2 inhibitors may prevent the onset of tumorigenesis in those at high risk for developing colorectal cancer and reduce the growth rate of established tumors, tumor regression is rarely observed. Despite these results obtained in FAP patients, Rofecoxib did not increase the antitumor activity of 5-fluorouracil combined with leucovorin, is associated with enhanced gastrointestinal toxicity (10), and had no short-term effect on secondary metastases (11).

Intrinsic or acquired resistance are important limitations to chemotherapeutic efficacy in the treatment of colorectal cancer. The use of death receptor ligands [belonging to the tumor necrosis factor (TNF) superfAMILY of ligands and the receptors] as therapeutic agents is therefore of potential importance, in particular because Fas-dependent component has been shown in the mechanism of 5-fluorouracil action (12). TNF-related apoptosis-inducing ligand (TRAIL), a member of the TNF family of ligands, induces apoptosis in a variety of transformed cells following ligation to TRAIL receptor-1 (TR-1/DR4) and/or TR-2/DR5 (13). Upon TRAIL-mediated DR4/DR5 receptor trimerization, the death-inducing complex (DISC) is formed, and cells consequently undergo TRAIL-mediated apoptosis by two differing mechanisms: type I, dependent of caspase-8 activation and independent of the mitochondria, or type II, requiring minimal caspase-8 activation with subsequent release of mitochondrial-related factors (14, 15).

TRAIL can induce apoptosis in transformed cells of diverse origin with little or no effects on normal cells (16). This is in contrast to Fas ligand (FasL), whose systemic use is limited by severe toxicity of multiple tissues, in particular, the liver (17). Furthermore, TRAIL exhibits antitumor activity in several xenograft studies in vivo without systemic toxicity (18, 19), and soluble Apo2L/TRAIL0 but not Apo2L/TRAIL.His seems nontoxic to liver cells (20). Based on these findings, clinical trials are currently in progress to assess the potential efficacy of TRAIL itself, or anti-DR4 and anti-DR5 antibodies, in treating human cancers. However, some tumor cell lines are resistant to TRAIL-induced apoptosis (21–24). Consequently, strategies that circumvent resistance to TRAIL-mediated apoptosis may further enhance the efficacy of TRAIL in the therapy of human cancers.
In the current study, we investigated the effect of specific COX-2 inhibitors on TRAIL-induced apoptosis in human colon carcinoma cells and have determined that the combination leads to a dramatic increase in the induction of apoptosis and a concomitant decrease in overall cell survival. The study has discovered a novel mechanism by which specific COX-2 inhibitors sensitize type I and II colon carcinoma cells to TRAIL, which involves ceramide-induced clustering of the DR5 receptor in cholesterol-rich domains identified as caveolae and redistribution of the DISC components into caveolae in type II cells.

Materials and Methods

Materials. DuP-697, arachidonic acid, and prostaglandin E2 (PGE2) enzyme immunoassay kit were from Cayman Chemicals (Ann Arbor, MI). Imipramine, methyl-β-cyclodextrin, and α-cyclodextrin were obtained from Sigma (St. Louis, MO); C6-ceramide and sphingomyelinase (from Staphylococcus aureus) were from Biomol (Plymouth Meeting, PA). N-methyl-[3H]-sphingomyelin was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO), and [3H]-arachidonic acid was purchased from Perkin-Elmer Life Sciences, Inc. (Boston, MA). Soluble TRAIL was prepared as previously described (15).

Cell culture. The HT29 human colon carcinoma cell line was obtained from American Type Culture Collection (Rockville, MD). GC2/c1 and VRC5/c1 were established in our laboratory as previously reported (25). RKO was obtained from Dr. Michael Kastan (St. Jude Children’s Research Hospital). Cells were maintained in the presence of folate-free RPMI 1640 containing 50 μg/ml of 5-methyltetrahydrofolate.

Measurement of cyclooxygenase-2 activity. Cells were seeded at a density of 1 x 10^6 per well in six-well plates and maintained overnight in culture medium. After 24 hours, cells were treated with vehicle or DuP-697 (50 μM/L) for 1 or 24 hours in serum-free medium containing arachidonic acid (25 μM/L). Supernatants were collected, and the PGE2 concentration was determined by an enzyme immunoassay according to the manufacturer’s protocol (Cayman Chemicals). The PGE2 concentration was normalized against the cellular protein content.

Clonogenic assays. Cells were plated at a density of 1,000 (HT29) or 3,000 (VRC5/c1 and GC2/c1) per well in six-well plates. After overnight attachment, cells were treated in triplicate with vehicle, TRAIL (0.1-50 ng/mL), DuP-697 (30 μM/L), or a combination of TRAIL and DuP-697 for 48 hours. After removal of the drug-containing medium, cells were allowed to regrow in fresh medium for 5 days (HT29) or 7 days (GC2/c1 and VRC5/c1), the equivalent of seven population doublings. Clonogenic survival was determined by counting residual clones using AlphaImager software as previously described (25).

Apoptosis assays. Cells were plated at a density of 200,000 per well in 12-well plates. After overnight attachment, cells were treated for 24 hours with either vehicle, TRAIL (1-100 ng/mL), DuP-697 (50 μM/L), or both agents. Where indicated, cells were pretreated for 20 minutes with methyl-β-cyclodextrin (1-5 mM/L), α-cyclodextrin (1-5 mM/L), 1 hour with imipramine (50 μM/L), 30 minutes with sphingomyelinase (500 milliunits/mL), or cotreated arachidonic acid (10-20 μM/L) or C6-ceramide (2.5 μM/L). Cells were detached in PBS/2 mM/L EDTA, centrifuged at 1,000 rpm for 5 minutes, and then gently resuspended in 200 μL hypotonic fluorochrome solution (200 μL PBS, 10 mg propidium iodide, 3.4 mM/L sodium citrate, 1 mM/L Tris, 0.1 mM/L EDTA, and 0.1% Triton X-100). The DNA content was analyzed by flow cytometry (Becton Dickinson FACScan, San Diego, CA). Twenty thousand events were analyzed per sample, and apoptosis was determined as the percentage of sub-G0/G1 DNA content.

Western blotting. After treatment, cells were detached in PBS/2 mM/L EDTA, centrifuged at 1,000 rpm for 5 minutes, and lysed in 50 μL of ice-cold lysis buffer [10 mM/L Tris (pH 7.6), 150 mM/L NaCl, 2 μg/mL aprotinin, 2 mM/L phenylmethylsulfonyl fluoride (PMSF), 5 mM/L EDTA, and 1% Triton X-100]. Protein concentrations were determined by the Bio-Rad protein assay (Hercules, CA) according to the manufacturer’s instructions; 50 μg of protein were electrophoresed by SDS-PAGE (Bio-Rad). Separated proteins were transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA). Blots were probed with anti-COX-1 and anti-COX-2 (Cayman Chemicals); anti-DR5 (Ab-1; Oncogene Research Product, San Diego, CA); anti-caspase-3 (31A1067; Alexis Biochemicals, San Diego, CA); anti-caspase-8 (S28), anti- XIAP, and anti-caspase-9 (S14; MBL, Watertown, MA); anti-PARP (C2-10), anti-FADD, and anti-Botulin (BD Transduction Laboratories, San Diego, CA); anti-caveolin-1 (N20; Santa Cruz Biotechnology, La Jolla, CA); anti-DR5 (Ab-1; Oncogene Research Product); and anti-β-actin (AC-40; Sigma) followed by horseradish peroxidase–conjugated antibodies (BD Transduction Laboratories). The enhanced chemiluminescence system (Pierce Biotechnology, Inc., Rockford, IL) followed by exposure to CL-Xposure films (Kodak, Rochester, NY) was used to visualize proteins.

Small interfering RNA system and transfection. Small interfering RNAs (siRNA) were synthesized by Dharmacon Research, Inc. (Lafayette, CO). The siRNA COX-2, actually consisted of a mixture of four siRNA duplexes targeting four different regions of COX-2 mRNA (siGENOME SMARTpool COX-2, M-004557-01). A pool of four nontargeting siRNA duplexes was used as a negative control (siCONTROL nontargeting siRNA pool, D-001206-13). All procedures were done in a RNase-free environment as described in detail by Zhang et al. (26). Transfection of cells with siRNA duplexes was done using TransIT-TKO transfection kits (Mirus Corp., Madison, WI). To determine the optimum conditions for COX-2 down-regulation, cells were transfected with 0, 25, and 100 mM/L of siRNA control and siRNA COX-2 for 72 hours as recommended by Dharmacon. The down-regulation of COX-2 was determined by Western blot. For apoptosis experiments, cells were transfected with 0, 25, and 100 mM/L of siRNA control and siRNA COX-2 for 72 hours without changing the medium and subsequently treated for 24 hours with vehicle or TRAIL (0.5-50 ng/mL).

Plasmid vectors and transfection. The human COX-2 cDNA (provided by Dr. Jeffrey J. Prusakiewicz, Vanderbilt University, Nashville, TN) was subcloned in the Sall/NotI sites of the retroviral expression vector pMSCV-I-GFP (provided by Dr. Peter McKinnon, St. Jude Children’s Research Hospital). Retroviral supernatants were prepared as described previously (27). Transfection of RKO and expression of COX-2 were confirmed by FACSscan and Western blot analysis.

Immunostaining of DR5, ceramide, and caveolin-1. Cells were treated with vehicle, DuP-697 (50 μM/L), or arachidonic acid (20 μM/L) for 0 to 6 hours and where indicated, cells were pretreated 1 hour with methyl-β-cyclodextrin or α-cyclodextrin (5 mM/L) or 1 hour with imipramine (50 μM/L). After treatment, adherent cells were washed in PBS and fixed for 10 minutes with PBS/4% paraformaldehyde on ice. For caveolin-1 staining, cells were permeabilized 30 minutes with PBS/0.02% Triton X-100. After three washes with PBS, cells were blocked 30 minutes with PBS/0.2% bovine serum albumin. Cells were washed three times with PBS/0.2% bovine serum albumin and incubated with anti-DR5 and/or anti-ceramide (MID15B4, Alexis Biochemicals) and/or caveolin-1 (2297, BD Transduction Laboratories) antibodies, diluted 1:100 (v/v) overnight at 4°C. After three washes, cells were incubated with the Alexa Fluor 488–conjugated goat anti-rabbit (Molecular Probes, Eugene, OR) and/or Cy3-conjugated goat antimouse (Jackson Research Laboratories, Inc., West Grove, PA) and/or Rhodamine Red-X-conjugated goat anti-mouse (Jackson Research Laboratories) antibodies at 1:200 (v/v) dilution for 2 hours at room temperature. When done, nuclei were stained with Hoechst at 1:10 (v/v) dilution for 1 hour at room temperature. Slides were finally sealed using mounting medium (Sigma). Images were obtained using a Zeiss axioskop2 microscope coupled to a spot digital camera (Diagnostic Instruments, Sterling Heights, MI) or a Bio-Rad 1024 MRC confocal microscope with a ×60 epifluorescence objective (Nikon, Tokyo, Japan). The percentage of DR5 that colocalized with ceramide or caveolin-1 was scored from a total of 100 cells by confocal microscopy. Clustering of DR5 with ceramide or caveolin-1 was defined as the percentage of cells displaying one or several intense and discrete fluorescent domains across the plasma membrane compared with those with a homogenous distribution of fluorescence.

Cell fractionation. Vehicle-treated and DuP-697-treated (50 μM/L) HT29 and GC2/c1 (100 x 10^6) washed in ice-cold PBS were lysed in 1 mL of
Cyclooxygenase-2 inhibition sensitizes to TRAIL-induced apoptosis

**Cyclooxygenase-2 Inhibition Sensitizes to TRAIL-Induced Apoptosis**

The long-term effect of COX-2 inhibition on TRAIL-mediated cytotoxicity was subsequently determined by clonogenic survival assay (Fig. 1D). TRAIL reduced clonogenic survival in both type I and II cells in a concentration-dependent manner. Although DuP-697 (30 μmol/L) alone did not significantly affect the loss of clonogenicity mediated by TRAIL treatment of GC3/c1, it synergistically potentiated TRAIL-induced loss of clonogenicity in VRC3/c1 and HT29.

Sensitization of TRAIL-induced apoptosis and cytotoxicity by DuP-697 was accompanied by activation of caspase-8 and caspase-3, reflected by the appearance of cleaved products, down-regulation of XIAP, and a cleavage of PARP in all cell lines at 6 hours (Fig. 1F). In agreement with Ozoren and El-Deiry (14), the cleavage of caspase-9 was only detected in type II cells. A complete cleavage of caspase-8, caspase-3, and PARP was observed after 24 hours (Fig. 1F).

Collectively, these data show that inhibition of COX-2 sensitized human colon carcinoma cells to TRAIL-induced apoptosis.

Cyclooxygenase-2 inhibition induces DR5 receptor clustering in cholesterol-rich and ceramide-rich domains in type I and II cells. To determine the mechanism of COX-2-mediated sensitization to TRAIL-mediated apoptosis, membrane expression of the TRAIL receptors DR4 and DR5 was initially analyzed by flow cytometry. All cell lines expressed DR5; however, DR4 was only weakly expressed. DuP-697 did not modify DR4 or DR5 expression. Furthermore, Western blot analysis of whole cell lysates from cells treated with DuP-697 revealed no modulation of DR5 levels (data not shown). These data indicate that DuP-697-mediated amplification of TRAIL-mediated apoptosis in human colon carcinoma cells was not due to increased receptor expression.

It has recently been shown that cell death mediated by stimulation of the related death receptor Fas requires the redistribution and the aggregation of Fas into ceramide-rich lipid platforms to form caps (30, 31). The forced clustering of Fas enhanced apoptosis mediated by FasL, indicating that the level of receptor membrane expression does not necessarily relate to function. We therefore did immunostaining of the strongly expressed membrane receptor DR5 and the sphingolipid ceramide. In vehicle-treated HT29 cells, diffuse DR5 and ceramide staining at the plasma membrane was observed with partial colocalization. Treatment of cells with DuP-697 (50 μmol/L) induced the rapid redistribution of DR5 and ceramide to concentrate and coalesce into large, polarized domains within the plasma membrane outer leaflet. This effect was initially observed within 15 minutes and was maintained for up to 6 hours (Fig. 2A). This observation was reflected in both VRC3/C1 (type II) and GC3/C1 (type I) cells following treatment with DuP-697 (data not shown). The redistribution and coalescence of DR5 with ceramide was confirmed by confocal microscopy of HT29 cells treated with DuP-697 (50 μmol/L), and the number of cells presenting DR5 and ceramide colocalized to enlarged, discrete domains quantified. DuP-697 (50 μmol/L) treatment induced a substantial increase in the percentage of HT29 cells processing discrete DR5 and ceramide coexisting with vehicle-treated control cells (Fig. 2B).

The involvement of COX-2 in the clustering of DR5 and the redistribution of ceramide was verified by immunostaining of siRNA-transfected cells. Although siRNAcontrol did not affect cell membrane localization of either DR5 or ceramide, siRNAcox−2 induced DR5 clustering in HT29 as well as ceramide redistribution into large platforms that overlap DR5 staining (Fig. 2C). Identical results were obtained by confocal microscopy and quantification showed 73 ± 1% DR5/ceramide colocalization in siRNAcox−2-treated cells versus 21 ± 5% in siRNAcontrol-treated cells (Fig. 2D).

In contrast, treatment of COX-2 nonexpressing RKO with DuP-697 (50 μmol/L) for 6 hours did not induce the appearance of DR5-enriched membrane domains (Fig. 2E). These results suggest that selective COX-2 inhibition leads to rearrangement of plasma...
membrane lipids leading to the clustering of DR5 into ceramide-enriched domains in COX-2-expressing cells.

Cholesterol and ceramide are essential in the clustering of DR5 and sensitization to tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis following cyclooxygenase-2 inhibition. It was recently shown that translocation of the ceramide-producing enzyme acid sphingomyelinase to the plasma membrane of T cells is required to induce the formation of ceramide-rich lipid platforms in which Fas aggregates to form a cap (30, 31). This clustering of DR5 to ceramide-enriched domains was completely abolished by the acid sphingomyelinase inhibitor imipramine. Imipramine alone (50 μmol/L) did not affect the redistribution of DR5 or ceramide staining (Fig. 3A). Furthermore, DuP-697 induced a rapid enhancement in acid sphingomyelinase activity in GC/c1, HT29, and VRC5/c1 (Fig. 3B) that corresponded with the observed redistribution of ceramide and DR5 at the plasma membrane (Fig. 3A). Enhanced acid sphingomyelinase activity was observed in all cell line within 15 minutes of DuP-697 (50 μmol/L) exposure and remained elevated above controls for remainder of the experimental period (Fig. 3B). Elevation in cellular ceramide through the application of bacterial sphingomyelinase (500 milli-units/mL) to induce ceramide formation from sphingomyelin, or exposure to C16-ceramide (2.5 μmol/L), enhanced apoptosis induced by TRAIL (1-10 ng/mL) in both cell types (Fig. 3C).

Loss of cholesterol is known to disrupt ceramide-enriched plasma membrane microdomains. The redistribution of both DR5 and ceramide was prevented by pretreatment with the cholesterol-depleting agent methyl-β-cyclodextrin (5 mmol/L; Fig. 3A) but not the steroisomer α-cyclodextrin (5 mmol/L; data not shown). Although β-cyclodextrin did not significantly affect apoptosis mediated either by TRAIL or by DuP-697 alone, it abolished the apoptosis (Fig. 3D), the activation of caspase-3, and cleavage of PARP induced by treatment with TRAIL and DuP-697 in combination (Fig. 3E). Overall, these data suggest that the clustering of DR5 receptors and the consequent sensitization to TRAIL-mediated apoptosis following COX-2 inhibition essentially require modulation of lipid metabolism at the plasma membrane.

Arachidonic acid mediates the redistribution of DR5 and sensitization of tumor necrosis factor–related apoptosis-inducing ligand–induced apoptosis following cyclooxygenase-2 inhibition. Because the COX family of enzymes uses arachidonic acid as a substrate to generate eicosanoids, we determined the involvement of arachidonic acid in the activation of acid sphingomyelinase, the sensitization of TRAIL-induced apoptosis following COX-2 inhibition, and finally the clustering of DR5. Inhibition of COX-2 activity with DuP-697 (50 μmol/L) induced an immediate and persistent elevation (within 15 minutes) in intracellular arachidonic acid in all three cell lines (Fig. 4A). Although the
exogenous exposure of colon carcinoma cells to arachidonic acid alone did not modulate apoptosis, it significantly enhanced the apoptosis induced by TRAIL (1-10 ng/mL) in all cell types (Fig. 4B) and rapidly enhanced acid sphingomyelinase activity (data not shown). Finally, the effect of arachidonic acid in mediating the biophysical redistribution of DR5 into cholesterol-enriched and ceramide-enriched domains was determined. In vehicle-treated cells, diffuse ceramide staining was observed at the outer leaflet of the plasma membrane that colocalizes with DR5. Following treatment with arachidonic acid (20 μmol/L, 6 hours), ceramide was redistributed into large platforms within the outer leaflet of the plasma membrane to overlap with DR5 caps (Fig. 4C). The clustering of DR5 to ceramide-enriched domains was completely abolished following cholesterol depletion using α-cyclodextrin and inactivation of acid sphingomyelinase using imipramine, indicating an essential requirement for ceramide production following activation of acid sphingomyelinase by arachidonic acid (Fig. 4C). α-Cyclodextrin did not inhibit arachidonic acid–mediated clustering of DR5 or redistribution of ceramide (data not shown). Similar results were observed in VRC5/c1 and GC5/c1 (data not shown).

**DuP-697 induces the redistribution of DR5 and death-inducing complex–forming molecules into caveolae.** DISC formation following DR5 receptor activation is essential for efficient propagation of the apoptosis signal mediated by TRAIL. Like DR5, we hypothesized that the distribution of DISC components within the plasma membrane may also be affected by COX-2 inhibition. A typical feature of lipid rafts is their resistance to extraction by the nonionic detergent Triton X-100 in a sucrose containing density gradient at 4°C. Thus, the plasma membranes of vehicle-treated and DuP-697-treated cells (6 hours) were collected in Triton X-100–containing buffer at low temperature and subjected to ultracentrifugation onto a linear sucrose gradient to collect 11 fractions. Each fraction was evaluated by Western blot for the presence of DR5, FADD, procaspase-8, flotilin (a raft-associated protein), and caveolin-1 (a caveolae-associated protein). In vehicle-treated HT29 (type II), DR5 was expressed in...
fractions 5 to 11, procaspase-8 was expressed in fractions 5 to 8, and expression of FADD was observed in fractions 8 to 11. These DISC components are distributed in resting HT29 cells in fractions positive for caveolin-1 (fractions 5, 6, 10, and 11) and flotilin (fractions 4-10), suggesting a disperse partition around the cell plasma membrane (Fig. 5A). However, when cells were treated with DuP-697, DR5, FADD, procaspase-8, and caveolin-1 were lost from fractions 5 to 8, subsequently to concentrate in fractions 9 to 11. No modification in flotilin expression was observed in DuP-697-treated HT29 cells compared with the vehicle-treated controls. In contrast, DR5, FADD, and procaspase-8 were specifically located in fractions 10 and 11 of GC3/C1 cells, positive for caveolin-1 but not flotilin (fractions 3-9). DuP-697 treatment of GC3/C1 did not affect the distribution of DR5, FADD, procaspase-8, flotilin, or caveolin-1. Together, these data suggest that DR5 and components of DISC are differentially expressed within the plasma membrane of type I cells compared with type II cells. We hypothesize that DR5 and components of DISC preexist within the same microdomains of the plasma membrane of type I cells, permitting efficient propagation of the death signal upon activation. Conversely, DR5, FADD, and procaspase-8 are expressed in various domains of the plasma membrane of type II cells, impractical for efficient DR5 signal propagation. COX-2 inhibition overcomes this inefficiency by organizing DR5 and its DISC components to exist within the same fractions of the plasma membrane of type II cells.

To confirm the redistribution of DR5 into caveolae following COX-2 inhibition, we did immunostaining of DR5 and caveolin-1. In vehicle-treated (Fig. 5B) or siRNAcontrol-transfected cells (Fig. 5C), DR5 staining was diffuse at the plasma membrane, whereas caveolin-1 staining was disperse within and at the plasma membrane of the cells. Treatment of cells with DuP-697 (50 μmol/L; Fig. 5B) or transfection of siRNACOX-2 (Fig. 5C) induced the rapid redistribution of DR5 to concentrate into large plasma membrane domains to colocalize with caveolin-1 (Fig. 5B and C). Quantification showed 78 ± 3% (for DuP-697-treated cells) and 72 ± 4% (in siRNACOX-2-transfected cells) colocalization of DR5 and caveolin-1 versus 6 ± 0% (for vehicle-treated cells) and 0 ± 0% (in siRNAcontrol-transfected cells; Fig. 5B and C).

Figure 2. COX-2 inhibition induces DR5 receptor clustering in ceramide-rich lipid platforms in type I and II cells. A, immunostaining of DR5 (green), ceramide (red), and nucleus (blue) in HT29 exposed to either vehicle or DuP-697 (50 μmol/L) for 15 minutes or 6 hours done by fluorescence microscopy. B, immunostaining of DR5 (green) and ceramide (red) in HT29 exposed to either vehicle or DuP-697 (50 μmol/L) for 6 hours done by confocal microscopy. % HT29 cells that possess distinct colocalization of DR5 and ceramide.

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Discussion

We have determined that COX-2 inhibition leads to sensitization of type I and II human colon carcinoma cells to TRAIL-mediated apoptosis via clustering of the TRAIL receptor DR5 in cholesterol-enriched and ceramide-enriched caveolae. These events essentially require the activation of acid sphingomyelinase mediated by the consequential accumulation of arachidonic acid following COX-2 inhibition. Reorganization of ceramide and DR5 within the plasma membrane of TRAIL-resistant type II cells following COX-2 inhibition is also associated with redistribution of FADD and procaspase-8 into caveolae from other membrane microdomains. Conversely, TRAIL-sensitive type I cells process both FADD and procaspase-8 exclusively located in caveolae.

Cells overexpressing COX-2 seem more resistant to the induction of apoptosis and also more resistant to chemotherapy (32, 33). COX-2 inhibitors have potential as radiosensitizers (34) and have also sensitized HeLa cells to Fas-induced and TNF-α-induced cell death (35); however, their efficacy to potentiate chemosensitivity is dependent upon the specific agent (10, 35–38). Accordingly, the present study showed that COX-2 inhibition leads to a dramatic...
sensitization to TRAIL-induced apoptosis in type I and II cells independently of p53 and further reduced clonogenic survival following TRAIL withdrawal.

Differing TRAIL sensitivities are observed in human colon carcinoma cells and have been primarily linked to membrane expression levels of DR4 and DR5 (35). Treatment with cytotoxic drugs, including COX-2 inhibitors, can result in the up-regulation of TRAIL receptors on the cell membrane, thereby sensitizing tumor cells to TRAIL (33, 39). However, no modification of the level of either DR4 or DR5 expression was detected in the current study, suggesting that the expression level was not involved in sensitization of these cells to TRAIL during COX-2 inhibition.

In addition to up-regulation of membrane receptors, it has recently been described that death receptor clustering in ceramide-rich lipid rafts or caveolae is critical for transmembrane signaling; furthermore, artificial clustering can amplify the death signal (40, 41). The acid sphingomyelinase–mediated generation of ceramide at the outer leaflet of the plasma membrane is essentially required for clustering of Fas receptors (31, 40). In the present study, fluorescent microscopy showed a homogeneous distribution of DR5 receptors that in part colocalize to small, ceramide-rich microdomains in vehicle-treated cells. In COX-2-inhibited cells, the DR5 microdomains coalesced towards specific domains or towards one pole of the cell to form caps of DR5 contained within large ceramide-rich lipid platforms. This effect was mimicked by exogenous application of arachidonic acid. These platforms were identified as caveolae by cell fractionation and confocal microscopy and have previously been reported to contain other death receptors, including Fas (40, 42).

In agreement with the findings of Totzke et al. (35), exposure to PGE2 did not inhibit the induction of apoptosis in cell lines treated with TRAIL alone, or in combination with DuP-697. Increased acid sphingomyelinase activity following DuP-697 treatment was rapid, initially observed within 15 minutes, and maintained above control treated cells for up to 6 hours. This is in contrast to the transient elevation in acid sphingomyelinase activity observed following death receptor stimulation (43) and is likely to be due to the persistent accumulation of arachidonic acid as a consequence of COX-2 inhibition by DuP-697. The data imply that it is the cellular

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**Figure 3.** Involvement of cholesterol and ceramide in clustering of DR5 and sensitization to TRAIL-induced apoptosis during COX-2 inhibition. A, immunostaining of DR5 (green), ceramide (red), and nucleus (blue) in HT29 exposed to either vehicle, DuP-697 (50 μmol/L), imipramine (50 μmol/L) alone or in combination of DuP-697, and methyl-β-cyclodextrin (β-CDXT, 5 mmol/L) alone or in combination with DuP-697 for 6 hours done by fluorescence microscopy.
accumulation of arachidonic acid and the consequential buildup in ceramide at the outer leaflet of the plasma membrane rather than loss of prostaglandin synthesis that sensitizes colon carcinoma cells to TRAIL. In addition, this biophysical reorganization in plasma membrane lipid structure was attenuated by the proteolytic degradation of acid sphingomyelinase in the presence of imipramine, suggesting an essential requirement for acid sphingomyelinase in the formation of the lipid platforms and DR5 capping. These observations are consistent with localization of the acid sphingomyelinase in caveolae (44), localization of the acid sphingomyelinase substrate sphingomyelin within the plasma membrane outer leaflet (45), and richness of caveolae for ceramide (46, 47). Cholesterol depletion, to disrupt sphingolipid microdomains, completely prevented DR5 capping and enhancement of TRAIL-mediated apoptosis. Similar to the effects of DuP-697, C16-ceramide, and bacterial SMase, arachidonic acid augmented TRAIL-induced apoptosis in all cell lines examined. Collectively, these data support the rearrangement of membrane lipid structure during COX-2 inhibition. Furthermore, our data suggest that the build up of cellular arachidonic acid mediates formation of large ceramide-rich lipid platforms in the outer plasma membrane leaflet, DR5 clustering, and sensitization to TRAIL-induced apoptosis during COX-2 inhibition in colon carcinoma cells.

Although TRAIL-induced capping of DR5 (data not shown) results in only minimal apoptosis, cells undergo complete apoptosis during cotreatment with DuP-697, suggesting that the intracellular apoptotic signaling pathway is not defective. Thus, insufficiency in receptor clustering and/or recruitment of intracellular signaling molecules to specific plasma membrane microdomains can explain the lack of sensitivity to TRAIL administered alone. Recently, Grassme et al. (48) reported that ligation of Fas in T cells led to limited DISC formation with as little as 1% caspase-8 activation, insufficient to mediate apoptosis. However, the subsequent formation of “rafts” seemed essential for the clustering of Fas, thereby functioning as a positive amplification mechanism for enhanced DISC formation and maximal apoptosis induction (48). Isolation of the plasma membrane lipid microdomains and consequential analysis of the proteins associated with the propagation of the

Figure 3

Continued. B, GC3/c1, VRC5/c1, and HT29 were exposed to vehicle or DuP-697 (50 μM) for 0 to 6 hours, and the ASM activity was quantitated. Points, mean of three independent experiments; bars, SE. C, apoptosis induction in GC3/c1, VRC5/c1, and HT29 following treatment for 24 hours with either vehicle, TRAIL (1-10 ng/mL), C16-ceramide (2.5 μM), and SMase (500 milliunits/mL), TRAIL and C16-ceramide, or TRAIL and SMase. Columns, mean of three to five independent experiments; bars, SE. *, P < 0.05; **, P < 0.01; ***, P < 0.001, significant. D, % apoptosis in GC3/c1, VRC5/c1, or HT29 following treatment for 24 hours with vehicle, DuP-697 (50 μM), TRAIL (1-10 ng/mL), or a combination of TRAIL and DuP-697 in the presence or absence of β-cyclodextrin (1-5 mmol/L). Columns, mean of three to five independent experiments; bars, SE. *, P < 0.05; **, P < 0.01, significant differences. E, GC3/c1, VRC5/c1, and HT29 were treated with vehicle, TRAIL (10 ng/mL), DuP-697 (50 μM), or TRAIL and DuP-697 in the absence or presence of β-cyclodextrin (5 mmol/L) and analyzed for PARP and caspase-3 expression. Representative of three to five independent experiments.
DR5 death signal (DR5, FADD, and procaspase-8) showed differential distribution between type I and II cells. TRAIL-resistant type II cells displayed widespread expression within between caveolae and noncaveolae lipid microdomains. Whereas in TRAIL-sensitive type I cells, DR5, FADD, and procaspase-8 were exclusively associated with caveolae. Upon COX-2 inhibition in TRAIL-resistant type II cells, caveolae recruit and concentrate DR5, procaspase-8, and FADD.

Together, our results show that COX-2 inhibition sensitizes colon cancer cells to TRAIL by a dual mechanism, one that is common to both type I and II cells, and a second that is applicable to type II cells only. (a) Following COX-2 inhibition, DR5, ceramide, and caveolae are redistributed and concentrated in specific, polarized domains of type I and II colon cancer cells. This process essentially requires the generation of arachidonic acid and activation of acid sphingomyelinase. (b) Whereas the plasma membrane of type I cells contain DR5, FADD, and procaspase-8 exclusively located in caveolae, type II cells show a disparate distribution of the DR5 DISC components within and outside caveolae. COX-2 inhibition
reorganizes the DR5, FADD, and procaspase-8 of type II cells to collectively associate within caveolae. We believe that these two processes prime the DR5 receptor for more rapid and efficient transduction of the death signal.

Multiple studies have shown that NSAIDs and specific COX-2 inhibitors effectively prevent the occurrence of colon carcinoma in animal models (49) and lead to the regression of colorectal polyps in patients with FAP and in patients with a family history of colon carcinoma. Reports from in vitro studies have described the proapoptotic effects of COX-2 inhibitors to be independent of their COX-2 suppressive function, thereby raising concerns that gastrointestinal complications associated with selective COX-1 inhibition or nonspecific COX inhibition may be reciprocated in vivo. The current demonstration that clustering of DR5 mediated through COX-2 inhibition can overcome resistance to TRAIL administration alone may provide significant potential for an effective therapeutic strategy in the treatment of colorectal carcinoma.

Figure 5. DuP-697 induces the redistribution of DR5 and DISC-forming molecules into caveolae. A, HT29 and GC/c1 cells were treated with either vehicle or DuP-697 (50 μmol/L) for 6 hours. Plasma membrane fractions were isolated and lipid microdomains were fractionated by centrifugation over a linear sucrose gradient. An equal volume of each collected fraction was submitted to SDS-PAGE before analysis of DR5, FADD, procaspase-8, caveolin-1, and flotilin expression by Western blot. One representative of three independent experiments. B, immunostaining of DR5 (green) and caveolin-1 (red) in HT29 exposed to either vehicle or DuP-697 (50 μmol/L) for 6 hours done by confocal microscopy. % Colocalization of DR5 and caveolin-1. C, immunostaining of DR5 (green) and caveolin-1 (red) in HT29 transfected either with siRNAcontrol or siRNA COX-2 (25 nmol/L) done by confocal microscopy. % Colocalization of HT29 cells that possess distinct DR5 and caveolin-1. Representative immunofluorescent images of three independent experiments. Columns, mean of five independent experiments; bars, SE. *** P < 0.001, significant.
In conclusion, this study has described for the first time that inhibition of COX-2 can sensitize type I and II human colon carcinoma cells to TRAIL-induced apoptosis via the capping of DR5 receptors and relocation of DisCl molecules in caveolae. This process essentially requires the accumulation of arachidonic acid and the activation of acid sphingomyelinase. Overall, the data presented provide substantial preclinical support for therapeutic strategies that specifically abrogate COX-2 activity in combination with TRAIL for the treatment of colon carcinoma.

References
Cyclooxygenase-2 Inhibition Sensitizes Human Colon Carcinoma Cells to TRAIL-Induced Apoptosis through Clustering of DR5 and Concentrating Death-Inducing Signaling Complex Components into Ceramide-Enriched Caveolae


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