The Chemopreventive Agent Sulindac Attenuates Expression of the Antiapoptotic Protein Survivin in Colorectal Carcinoma Cells

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ABSTRACT

Nonsteroidal anti-inflammatory drugs (NSAIDs) such as sulindac have chemopreventive activity against colorectal tumors. Although the molecular mechanism has not been fully established, it is thought to involve the ability of NSAIDs to induce apoptosis. Because the majority of colon carcinomas are known to overexpress antiapoptotic proteins such as survivin and Bcl-2 and show only limited ability to undergo apoptosis, we hypothesized that the ability of sulindac to cause regression of adenomas and to inhibit colon carcinogenesis is mediated, at least in part, by down-regulation of one or more of these antiapoptotic proteins. To test this hypothesis, we exposed HT-29 colon carcinoma cells to sulindac. Sulindac induced a sustained decrease in mRNA and protein expression for survivin but not for Bcl-2. This finding suggests that sulindac is selectively acting through a survivin-related pathway. This is consistent with our earlier finding that inhibition of the β-catenin:T-cell factor 4 (Tcf-4) pathway by the adenomatous polyposis coli protein down-regulates survivin expression and with recent evidence that sulindac induces β-catenin degradation, which would reduce Tcf-4 activation. This suggests that the β-catenin:Tcf-4:survivin mechanism may be a useful target for therapy or chemoprevention of colon cancer.

Several lines of evidence, including human epidemiological data and clinical intervention data, indicate that nonsteroidal anti-inflammatory drugs (NSAIDs) have chemopreventive activity against colorectal cancer (Fournier and Gordon, 2000; Patrignani 2000). The research includes numerous case control and cohort studies that show an inverse relationship between aspirin intake and the incidence of colorectal cancer: with aspirin use, the incidence of cancer decreases. NSAIDs such as sulindac have been shown to cause regression of adenomas in familial adenomatous polyposis (FAP) patients, to inhibit colon carcinogenesis in a rodent model, and to inhibit adenoma formation in the ApcMin mouse, an animal model of human FAP (Taylor et al., 2000; Torrance et al., 2000; Marx 2001).

The molecular mechanisms for sulindac’s antitumor effects have not been fully elucidated, although several have been described (He et al., 2000; Zhang et al., 2000; Marx, 2001). One molecular mechanism appears to involve sulindac’s ability, via its sulfide metabolite, to inhibit the enzymatic activity of both COX-1 and COX-2 and thereby inhibit prostaglandin synthesis. However, the effects of another metabolite, the sulfone product, appear to be COX-independent.

A cellular mechanism attributed to sulindac’s antitumor effects is its ability to induce apoptosis and decrease cell proliferation (Masunaga et al., 2000; Brown et al., 2001a). Indeed, the majority of cancers, including colon cancer, show only limited ability to undergo apoptosis and are known to overexpress the antiapoptotic protein survivin. Survivin is an inhibitor of apoptosis protein that is thought to contribute to tumor cell immortality. This protein may also be a key contributing factor in colon carcinogenesis. In a recent study we provided evidence indicating that APC suppresses survivin expression in the colonic crypt (Zhang et al., 2001). APC mutations are known to cause colorectal cancer, and they might do so by de-repressing the expression of survivin and thereby inhibiting apoptosis (Boman et al., 2004). In this view, the inability of cells with mutant APC to shut down the antiapoptotic effect of survivin expression contributes to tumorigenesis in the colon, although the underlying molecular mechanism has not been established.

We hypothesized that the ability of sulindac to cause re-
expression of adenomas and to inhibit colon carcinogenesis is mediated, at least in part, by down-regulation of the expression of antiapoptotic proteins. To test this hypothesis, we evaluated whether sulindac down-regulates the expression of Bcl-2 or survivin.

Materials and Methods

Cell Culture and Treatment. The colon carcinoma cell line HT-29 (American Type Culture Collection, Manassas, VA) was cultivated in McCoy's 5A medium (Cellgro; Mediatech, Herndon, VA) and supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 mg/ml streptomycin. The cells were treated with sulindac (200 μM) and sampled for up to 48 h.

We exposed HT-29 cells to a sulindac concentration of 200 μM because that is the concentration that completely reverses the cellular phenotype caused by stabilized/mutant β-catenin in vitro (Naishiro et al., 2001) and because this concentration is also higher than that achieved clinically (Mattila et al., 1984; Ravis et al., 1993) at the sulindac dose used to treat FAP patients (Giardiello et al., 1993). This concentration was also selected because it is below the sulindac concentration that results in apoptosis of HT-29 cells (Shiff et al., 1995); higher sulindac concentrations and the resultant cell death might nonspecifically decrease the level of survivin or bcl-2. All experiments were repeated at least three times on different days and with different cultures.

RNA Extraction and cDNA Synthesis. Total RNA was isolated from HT-29 cells before and after treatment with sulindac. Isolation was done using an RNeasy Mini Kit (QIAGEN, Valencia, CA) in accordance with the manufacturer’s instructions. The first strand of cDNA was synthesized from RNA using avian myeloleukemia virus reverse transcriptase as indicated by the manufacturer (Promega, Madison, WI). One microgram of RNA was used as a template for first-strand synthesis in quantitative analysis of mRNA expression.

RT-PCR Amplification. Survivin mRNA expression was evaluated semiquantitatively using RT-PCR. cDNA was made by reverse transcription with random primers. The primers used to detect fragments of the survivin gene were designed from published human sequences and span exons 1 to 4. The sequences were: 5′- AGCCCTT-TCTCAAGGACCA C′ 3′ and 5′- GACCTTCTCCGAGT TCC 3′, giving an amplified product of 363 base pairs. The PCR reaction contained 2 μl of Taq polymerase (Boehringer Mannheim, Indianapolis, IN); 10× PCR buffer, 10 pmol of each oligonucleotide primer; a 250 μM concentration, each, of dATP, dCTP, dGTP, and dTTP; 1 μl of nascent cDNA; and sterile distilled water to bring the volume to 25 μl. The amplification cycle included a denaturation step of 94°C for 2 min. This was followed by 28 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and concluded with a final primer extension step of 72°C for 5 min. Controls included replacing RNA or cDNA with distilled water. Controls were consistently found to be negative for survivin expression. PCR products were resolved in a 1.5% agarose gel in TAE buffer (Tris acetate EDTA) and visualized by ethidium bromide staining under UV illumination and photographed. To confirm the integrity of cDNA and to confirm equal loading on gels, the housekeeping gene β-actin was amplified concurrently. The survivin cDNA product was sequenced and confirmed to be survivin according to the known survivin sequence in GenBank.

Western Blot Analysis. HT-29 cells were lysed by 1% sodium dodecyl sulfate “running” buffer (100 mM Tris Cl, 200 mM dithiothreitol, 4% sodium dodecyl sulfate, 20% glycerol). The amount of protein in cell lysates was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA). After boiling for 10 min, 50 μg of protein was loaded and resolved by electrophoresis (12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The protein was then transferred to a nitrocellulose membrane by capillary action. The membrane was blocked for 30 min in blocking buffer (trizma-buffered saline with 0.2% Tween 20 and 5% nonfat milk) and then incubated overnight at 4°C with rabbit anti-human survivin polyclonal antibody (1:1500) or with Bcl-2 polyclonal antibody (1:1000); Santa Cruz Biotechnology, Inc., Santa Cruz, CA). The membrane was then washed in trizma-buffered saline containing 0.2% Tween 20, incubated with phosphatase-conjugated goat anti-rabbit antibody (1:1500) for 60 min, and developed with a substrate reagent kit (Bio-Rad). As a control, tubulin protein was blotted concurrently. Anti-survivin antibody was kindly supplied by Prof. Dario Altieri (Department of Pathology, Yale University, New Haven CT).

Densitometry. Quantitation of survivin mRNA and protein was done by densitometric scanning of photographs of the ethidium bromide-stained gels from our RT-PCR experiments and of Western blots. This was done using a Gateway 2000 Computer (G6-200XL), a Hewlett-Packard ScanJet 5P Scanner, and UN-SCAN-IT scanning software (Automated Digitizing System; Silk Scientific Inc., Orem, UT). The pixel total percentage was recorded, which is 100 multiplied by (pixel total)/(sum of all the pixel total – background) for that lane. Pixel total is the total sum of all the pixels within that band region.

Results

Effects of Sulindac on Expression of Survivin and Bcl-2 mRNA. Figure 1 shows a representative time course for survivin and Bcl-2 mRNA expression following exposure

![Figure 1](image)
of HT-29 cells to sulindac. Survivin expression was decreased at all times following sulindac treatment. For Bcl-2 expression, we observed a transient decrease followed by a steady increase up to 36 h. We found that the transient decrease was maximal at 8 h.

**Effects of Sulindac on Expression of Proteins for Survivin and Bcl-2.** Figure 2 shows a representative time course for survivin and Bcl-2 protein expression following exposure of HT-29 cells to sulindac. Survivin protein expression showed a monotonic decrease up to 36 h. For Bcl-2 protein expression we observed a slight (~14%), transient decrease at 12 h followed by a return to baseline.

**Discussion**

Using two different endpoints, RNA and protein, our results show that sulindac induces a sustained decrease in survivin expression. This effect of sulindac appears to be specific to survivin since Bcl-2, another antiapoptotic protein, was not similarly affected by sulindac. Survivin mRNA decreased maximally between 36 and 48 h with an average maximum decrease of 57% (±9%). The survivin protein decreased maximally between 36 and 48 h with an average maximum decrease of 69% (±4%). The timing of these decreases is consistent with the idea that the decrease in survivin mRNA causes the decrease in survivin protein.

Since survivin is known to be an antiapoptotic protein, our findings that sulindac decreases survivin expression are consistent with previous reports showing that sulindac increases apoptosis (Masunaga et al., 2000; Brown et al., 2001a). The lack of a sustained effect of sulindac on Bcl-2 expression is consistent with similar findings, as reported in two recent studies (McEntee et al., 1999; Zhang et al., 2000).

Because the majority of colon carcinoma cells are known to overexpress survivin (Ambrosini et al., 1997; Adida et al., 1998) and to show only limited ability to undergo apoptosis, our finding that sulindac decreases the expression of this antiapoptotic factor in human colon carcinoma cells provides a target for possible new therapeutic approaches to colorectal cancer treatment. Indeed, the ability of NSAIDs to increase apoptosis through decreases in survivin may be responsible for NSAID-induced regression of colonic adenomas (Masunaga et al., 2001), apoptosis and inhibition of growth in colon tumor cells lines (Richter et al., 2001), and inhibition of intestinal tumorigenesis in the Apc<sup>Min</sup> mouse (Beazier-Barclay et al., 1996). It should be noted, however, that although the drug has proven efficacy in reducing polyp size and number in FAP patients (Waddell and Loughry, 1983; Giardiello et al., 1993), it is not ideal because 1) tolerance can develop, and 2) sulindac does not appear to be effective against the development of new adenomas in FAP (Giardiello et al., 2002).

How sulindac regulates survivin expression is not fully established. One possible mechanism is that sulindac increases expression of APC (Schnitzler et al., 1996; Kishimoto et al., 2000) and that APC decreases survivin expression because, as we have shown (Zhang et al., 2001), APC down-regulates survivin through the β-catenin:Tcf-4 pathway. However, HT-29 cells lack wild-type APC, which excludes this possibility. A more likely possibility based on recent evidence is that sulindac and its metabolites induce, through a COX-independent mechanism, caspase- and proteasome-dependent degradation of β-catenin protein in human colon cancer cells (Mahmoud et al., 1997; McEntee et al., 1999; Thompson et al., 2000; Brown et al., 2001b; Li et al., 2002; Rice et al., 2003), and degradation of β-catenin would reduce Tcf-4 activation, which would reduce survivin expression and induce apoptosis.

Survivin is also known to promote cell division through activation of Aurora-b kinase, which regulates chromosome segregation and cytokinesis (Bolton et al., 2002; Chen et al., 2003). This suggests that the down-regulation of survivin expression by sulindac will also inhibit chromosome segregation and block cell division. Thus, sulindac might reduce growth of adenomas in FAP patients through both survivin-related mechanisms: induction of apoptosis and inhibition of cell division.

That sulindac might act therapeutically through more than one survivin-related mechanism is not surprising since analysis of survivin’s promoter region shows the presence of sequences in addition to the one for Tcf-4 that regulate survivin expression, suggesting that there may be multiple mechanisms that regulate survivin transcription. One such mechanism appears to involve wild-type p53 that negatively regulates survivin expression (Hoffman et al., 2002; Mirza et al., 2002; Zhou et al., 2002). However, in the case of sulindac’s ability to down-regulate survivin in HT-29 cells, this is not a likely mechanism because HT-29 cells contain mutant p53.

The above β-catenin:Tcf-4:survivin-related mechanisms, coupled with reports that sulindac affects other cellular pathways (Marx, 2001), suggests that sulindac’s clinical antitu-
mor effects may involve multiple cellular mechanisms. Nevertheless, the mechanism examined on here, sulindac regulation of survivin expression, might be particularly important inasmuch as it directly involves the main pathway, dysregulation of APC-β-catenin:Tcf-4 signaling, by which most colonic tumors appear to be initiated. Also, there may be other Tcf-4 target genes, in addition to survivin, that are affected by sulindac’s effects on β-catenin. Further work may identify specific steps in this pathway that could serve as effective targets for cancer treatment and even chemoprevention.

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References


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