The Chemopreventive Agent Sulindac Attenuates Expression of the Anti-Apoptotic Protein Survivin in Colorectal Carcinoma Cells.

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Abbreviations Used: APC, adenomatous polyposis coli; FAP, familial adenomatous polyposis; IAP, Baculovirus Inhibitor of Apoptosis Proteins Gene; NSAID, non-steroidal anti-inflammatory drug; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; RT-PCR, reverse transcription polymerase chain reaction; TBS, trizma-buffered saline; CRC, colorectal cancer;

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Abstract

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 anti-apoptotic 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 anti-apoptotic 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 the APC:beta-catenin:Tcf-4 pathway down-regulates survivin expression and with recent evidence that sulindac induces beta-catenin degradation which would reduce Tcf-4 activation. This suggests that the beta-catenin:Tcf-4:survivin mechanism may be a target for therapy or chemoprevention of colon cancer.
Several lines of evidence, including human epidemiological data and clinical intervention data, indicate that non-steroidal anti-inflammatory drugs (NSAIDs) have chemopreventive activity against colorectal cancer (Fournier & 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 \textit{Apc}^{Min} mouse, an animal model of human FAP (Marx 2001; Torrance et al., 2000; Taylor et al., 2000).

The molecular mechanisms for sulindac’s anti-tumor effects have not been fully elucidated, although several have been described (Marx, 2001; He et al., 2000; Zhang et al., 2000). 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 anti-tumor effects is its ability to induce apoptosis and decrease cell proliferation (Brown et al., 2001a; Masunaga et al., 2000). Indeed, the majority of cancers, including colon cancer, show only limited ability to undergo apoptosis and are known to overexpress the anti-apoptotic protein survivin. Survivin is an inhibitor of apoptosis protein (IAP) 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 showed that \textit{APC} suppresses survivin expression in the colonic crypt (Zhang et al., 2001). \textit{APC} mutations are
known to cause colorectal cancer and they might do so by de-repressing the expression of survivin and thereby inhibiting apoptosis. In this view, the inability of cells with mutant \( APC \) to shut down the anti-apoptotic 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 regression of adenomas and to inhibit colon carcinogenesis is mediated, at least in part, by down regulation of the expression of anti-apoptotic proteins. To test this hypothesis, we evaluated whether sulindac down-regulates the expression of Bcl-2 or survivin.
Methods

Cell culture and treatment

The colon carcinoma cell line HT-29 (ATCC, Manassas, VA) was cultivated in McCoy’s 5A medium (Cellgro, Herndon, VA), and supplemented with 10% fetal bovine serum, 100 unit/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 uM because (a) that is the concentration that completely reverses the cellular phenotype caused by stabilized/mutant beta-catenin \textit{in vitro} (Naishiro et al, 2001) and (b) it 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, 2002), which leads to decreased beta-catenin levels \textit{in vivo} (Keller et al, 2001) and reversal of the polyposis phenotype (Giardiello et al, 1993; Wadell & Loughery, 1983). 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 non-specifically 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 AMV 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 semi-quantitatively 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 1-4. The sequences were: 5’AGCCCTTTCTCAAGGACCAC 3’ and 5’GCACTTTTCTTCGCAGTTTCC 3’, giving an amplified product of 363 bp. The PCR reaction contained 2 units of Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN), 10X PCR buffer, 10 pmol of each oligonucleotide primer, 250 uM each of dATP, dCTP, dGTP, dTTP, 1 uL of nascent cDNA, and sterile distilled water to bring the volume to 25 uL. 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, 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 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 1X sodium dodecyl sulfate “running” buffer (100 mM TrisCl, 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 mg protein was loaded and resolved by electrophoresis (12% sodium dodecyl sulfate/PAGE). The protein was then transferred to a nitrocellulose membrane by capillary action. The membrane was blocked for 30 min in blocking buffer (TBS with 0.2% Tween20 and 5% nonfat milk), and then incubated overnight at 4°C with rabbit anti-human survivin polyclonal antibody (1:500) or with Bcl-2 polyclonal antibody (1:1000, Santa Cruz Technology, Santa Cruz, CA). The membrane was then washed in TBS containing 0.2% Tween20, incubated with phosphatase-conjugated goat anti-rabbit antibody (1:1500) for 60 min, and developed with a substrate reagent kit (Bio-Rad, Hercules, CA). 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 were 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, Utah). The pixel total percent 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 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 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 anti-apoptotic protein, was not similarly affected by sulindac. Survivin mRNA decreased maximally between 12 and 36 hours with an average maximum decrease of 57% (± 9%). The survivin protein decreased maximally between 36 and 48 hours 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 anti-apoptotic protein, our findings that sulindac decreases survivin expression are consistent with previous reports showing that sulindac increases apoptosis (Brown et al., 2001a; Masunaga et al., 2000). The lack of a sustained effect of sulindac on Bcl-2 expression is consistent with similar findings, as reported in two recent studies (Zhang et al., 2000; McEntee et al., 1999).

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 anti-apoptotic 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 ApcMin mouse (Beazer-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 & Loughry, 1983; Giardello et al., 1993), it is not ideal because (i) tolerance can develop, and (ii) sulindac does not appear to be effective against the development of new adenomas in FAP (Giardella 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 downregulates survivin through the APC:beta-catenin:Tcf-4 pathway. However, HT29 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 beta-catenin protein in human colon cancer cells (Rice et al., (in press); Thompson et al., 2000; Li et al., 2002; McEntee et al., 1999; Mahmoud et al., 1997; Brown et al., 2001b). And degradation of beta-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 downregulation 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 as 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 (Zhou et al., 2002; Hoffman et al., 2002; Mirza et al., 2002). However, in the case of sulindac's ability to down-regulate survivin in HT29 cells, this is not a likely mechanism because HT29 cells contain mutant P53.

The above beta-catenin:Tcf-4:survivin related mechanisms, coupled with reports that sulindac affects other cellular pathways (Marx 2001), suggests that sulindac's clinical anti-tumor effects may involve multiple cellular mechanisms. Nevertheless, the mechanism reported on here – sulindac regulation of survivin expression – might be particularly important as it directly involves the main pathway, dysregulation of APC:beta-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 beta-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.


Footnotes.

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Figure Legends.

**Figure 1.** Effects of sulindac on expression of survivin and Bcl-2 RNA. The figure, which represents results from densitometry done on ethidium bromide stained gels, shows a sustained decrease in survivin but not Bcl-2 RNA expression following exposure of HT-29 cells to sulindac. Survivin expression was substantially decreased at all times following sulindac treatment. For Bcl-2 expression the decrease was small and transient (8 h only) and was followed by a slight increase. Panel A shows typical gels for survivin and Bcl-2.

**Figure 2.** Effects of sulindac on expression of survivin and Bcl-2 proteins. The figure, which represents results from densitometry done on western blots, shows that sulindac induced a monotonic decrease in survivin but not Bcl-2 protein expression in HT-29 cells. For Bcl-2 protein expression we observed a slight, transient decrease (12 h) followed by a return to baseline. Panel A shows typical western blot results for survivin and Bcl-2.
Figure 1

A

Survivin

Bcl2

B

Percent of Control

Sulindac Treatment (hours)

Figure 1
Figure 2

(A) Western blot analysis of Survivin and Bcl2 proteins at different time points (0h, 12h, 24h, 36h).

(B) Graph showing the percent of control of Bcl2 and Survivin proteins over time with Sulindac treatment (hours).