Advances in Brief

Induction of Apoptotic Cell Death in Human Colorectal Carcinoma Cell Lines by a Cyclooxygenase-2 (COX-2)-selective Nonsteroidal Anti-Inflammatory Drug: Independence from COX-2 Protein Expression

Douglas J. E. Elder,2 Dawn E. Halton, Angela Hague, and Christos Paraskeva
Department of Pathology and Microbiology, School of Medical Sciences, University of Bristol, University Walk, Bristol, BS8 1TD, United Kingdom

Abstract
Cyclooxygenase (COX) catalyzes the conversion of arachidonic acid to prostaglandin H2. The inducible isoform, COX-2, promotes colorectal tumorigenesis, and nonsteroidal anti-inflammatory drugs (NSAIDs) that selectively inhibit this isoform are chemopreventive in murine models of intestinal tumorigenesis. To establish a mechanism for their chemopreventive properties, we examined the effect of a COX-2-selective inhibitor, NS-398, on two colorectal carcinoma cell lines: HT29, which was found to express COX-2 protein constitutively; and S/KS, which did not express detectable levels of COX-2 protein. NS-398 had a dose-dependent antiproliferative effect on each cell line (IC50, 82.0 ± 10.1 μM for HT29 and 78.6 ± 11.1 μM for S/KS), and this was due to the induction of apoptosis. Cell cycle parameters were unaffected by NS-398 treatment. The ability of NS-398 to induce apoptosis provides a potential mechanism by which COX-2-selective inhibitors are chemopreventive and also indicates their potential as chemotherapeutic agents for colorectal cancer. That this effect was independent of COX-2 protein expression suggests that COX-2-selective NSAIDs may, like nonselective NSAIDs, be antineoplastic in the absence of COX-2.

Introduction
There now exists a considerable body of evidence from epidemiological, clinical, animal, and in vitro studies to indicate that NSAIDs prevent colorectal cancer (1). This is important because this malignancy is one of the most common in the westernized world and remains largely resistant to current therapeutic strategies. Several recent in vitro and in vivo studies have indicated that the predominant effect of the active metabolites of aspirin and sulindac upon colorectal tumor cells is to induce apoptosis (2-4). This is a recognized mechanism of action of chemopreventive and chemotherapeutic agents and explains, at least in part, their chemopreventive effect. Unfortunately, these “traditional” NSAIDs have gastrointestinal side effects that are serious enough to restrict their use as chemopreventive agents (5). The adverse side effects result largely from the ability of traditional NSAIDs to inhibit COX-1. COX-1 is an isof orm of COX, a key enzyme in prostaglandin biosynthesis, and is expressed constitutively in many mammalian tissues (6). A second isof orm (COX-2), which is induced by cytokines, mitogens, endotoxins, and serum and is not detectable in most tissues under physiological conditions, has also been identified (7). Traditional NSAIDs, such as aspirin and sulindac, selectively inhibit COX-1. Newer NSAIDs that selectively inhibit COX-2 allow the production of physiologically important prostaglandins by COX-1 and as a result have very low gastrointestinal toxicity (6). Hence, when considering the chemoprevention of colorectal cancer, a crucial question concerns whether COX-2-selective NSAIDs complement their low gastrointestinal toxicity by having the antineoplastic properties of the traditional NSAIDs. Studies indicating that the overexpression of COX-2 in rat intestinal epithelial cells confers a more transformed cellular phenotype (8) and that COX-2 is overexpressed in most colorectal carcinoma tissue (7) provided early suggestions that the inhibition of COX-2 activity may be chemopreventive. Now, three recent studies have indicated that, like traditional NSAIDs, COX-2-selective NSAIDs are chemopreventive in murine models of colorectal tumorigenesis. Nim esulide (an analogue of NS-398) and SC-58635 inhibit the formation of chemically induced aberrant crypt foci in the rat colon, and MF tricyclic suppresses polyp formation in Apc5/1616 mice, which model human familial adenomatous polyposis (9-11). The latter study using Apc5/1616 mice also showed that a Pigs2 (mouse COX-2) null mutation dramatically reduced the number and size of the intestinal polyps that develop spontaneously in these mice (11). This is direct genetic evidence that COX-2 plays a key role in intestinal tumorigenesis and further indicates COX-2 activity as an effective target for chemoprevention.

None of these studies investigated cellular responses to the COX-2-selective inhibitors that would indicate a mechanism of chemoprevention. Because there is considerable evidence to indicate that the chemopreventive effects (such as the induction of apoptosis) of the traditional NSAIDs are not necessarily mediated through their property of inhibiting the COX isoforms, it cannot be assumed that the chemopreventive mechanisms of

Received 5/16/97; revised 7/22/97; accepted 7/24/97.
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1 This work was supported by a program grant from the Cancer Research Campaign of Great Britain.
2 To whom requests for reprints should be addressed. Phone: 44(0)117 9287892; Fax: 44(0)117 9287896.
3 The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase.
COX-2-selective NSAIDs are the same as those of traditional NSAIDs (12, 13). Also, it is unclear whether COX-2-selective inhibitors are antineoplastic in the absence of COX-2 protein or in human cells. Thus, two important questions remain unanswered. Do COX-2-selective NSAIDs retain the ability of the traditional NSAIDs to induce apoptosis in human colorectal tumor cells? Are effects of COX-2-selective inhibitors on human colorectal tumor cells dependent on the expression of COX-2 protein? To address these questions, we investigated the response of human colorectal carcinoma cells, with and without detectable COX-2 protein, to treatment with the COX-2-selective inhibitor NS-398. The data, showing growth inhibition by the induction of apoptosis, are the first to provide a potential mechanism for the chemopreventive effect of COX-2-selective NSAIDs and also show that this effect is not dependent on the expression of COX-2 protein. This further indicates the potential of these agents as chemopreventives for colorectal cancer.

Materials and Methods

Cell Lines and Culture Conditions. The cell lines used in this study were: HT29, which is derived from a human sporadic colon carcinoma; and S/KS, which is derived from a human sporadic rectal carcinoma (14). Each cell line was grown on tissue culture plastic in DMEM (Life Technologies, Inc.) with 20% fetal bovine serum (batch selected), as described previously (3).

Treatment with NS-398. Exponentially growing cells were trypsinized and then seeded at a density so that there were 2–3 × 10^6 cells/T25 flask at the start of treatment (3 days after seeding). NS-398 (Cayman Chemical Co., Ann Arbor, MI) was made up as a 13.3 mM stock solution in ethanol. The required final concentrations of NS-398 were prepared by diluting aliquots of this stock solution in medium, together with an appropriate volume of ethanol, so that each control and treated flask received an equal final volume of ethanol (0.8% v/v). Cells were treated in triplicate with up to 100 μM NS-398 for 96 h, and then the attached cells (those remaining adhered to the tissue culture flask) and the floating cells (those having detached from the adhered monolayer) were counted separately and stained with acridine orange for analysis by fluorescence microscopy (see below). IC_{50}s were determined from dose-response curves of the NS-398 concentration versus attached cell yield following a 96-h treatment.

Measurement of Apoptosis. As we and others have described previously, the level of apoptosis in cultured epithelial cell lines can be assessed by measuring the proportion of cells that have detached from the cell monolayer and are floating in the medium and by determining the fraction of these “floating” cells that are apoptotic (3, 8, 14). In the routine culture of colorectal epithelial tumor cells, the majority of spontaneously occurring floating cells have been shown to be apoptotic, whereas the proportion of attached cells that are apoptotic is low (<3%; Ref. 14). Treatment with agents such as salicylate or butyrate results in an increase in the proportion of cells that are floating, and this is found to be due to the induction of apoptosis (3, 14). Following NS-398 treatment, for each NS-398 dose the proportion of the total cell population that was floating was determined. To see if the induction of floating cells was due to apoptosis, the proportion of the attached and floating cell populations that were apoptotic was determined by staining with acridine orange (5 μg/ml in PBS) and analysis by fluorescence microscopy as described previously (14). Analysis was carried out by an experienced observer unaware of the cell type or treatment. Apoptotic cells were identified on the basis of well-documented morphological criteria, most obviously by their characteristically condensed chromatin stained by the acridine orange. Acridine orange-stained cells were photographed using Fujichrome Provia (ISO 400) film.

Analysis of Cell Cycle Distribution. The cell cycle phase distribution of the attached cells of each cell line was determined for control and treated flasks (20, 50, 75, and 100 μM) at the end of treatment (96 h) and for control flasks and those treated with 100 μM NS-398 at 24, 48, and 72 h following the initiation of treatment. In all cases, samples of attached cells were fixed with 70% ethanol and stained with 20 μg/ml propidium iodide (Sigma Chemical Co., Poole, United Kingdom) before being analyzed for red (FL2) fluorescence on a FACScan (Becton Dickinson), as detailed previously (15). The cell cycle distribution was calculated from the resultant DNA histograms using Cell FTI software, based on a rectangular S-phase model.

SDS-PAGE Western Blotting. Cell lysates were prepared, and SDS-PAGE Western blotting was carried out as described previously (16). Proteins from lysates of 10^6 cells were resolved in a 12.5% acrylamide gel and electroblotted onto polyvinylidene fluoride membrane, which was probed with monoclonal anti-cyclooxygenase-2 (0.2 μg/ml; Transduction Laboratories, Lexington, KY) and controlled for loading and transfer by repeat probing with anti-α-tubulin (Sigma) at 1:700 dilution. Native COX-2 protein (1 μg; Oxford Biomedical Research, Inc.) was used as a positive control for COX-2 protein. Proteins were detected using enhanced chemiluminescence (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Results and Discussion

Expression of COX-2. In this study, two colon carcinoma cell lines were used, HT29 and S/KS. Western immunoblotting analysis of crude cell lysates using a COX-2 antibody indicated that HT29 cells constitutively express COX-2 protein, whereas S/KS cells do not express levels detectable by this method (Fig. 1A). COX-2 expression in the HT29 cells reflects the situation that is becoming increasingly apparent from in vitro and in vivo studies. That is, in humans COX-2 protein is undetectable or expressed at very low levels in normal colonic mucosa but is readily detectable in most colorectal carcinoma tissue samples, where it predominates in the epithelial tumor cells (17–20). Consistent with this, a study of Min mouse intestinal adenomas has also shown elevated COX-2 expression, which predominates in the epithelial cells (21). However, it should be noted that one deviation from this generality was seen to occur in the intestinal polyps of Apc^{△716} mice (11). Here again, COX-2 expression was up-regulated in the intestinal adenomas but was localized to the interstitial cells rather than the epithelial cells (11). The significance of this result remains to be established, but the weight of evidence at present indicates the overexpression of COX-2 in colorectal tumors to be mainly in the epithelial cells. The lack of detectable COX-2 protein in...
cell lines and the effect of the COX-2-selective inhibitor NS-398 on ± 10.1 B described in their spontaneous levels of apoptosis were 5.6 respective control (spontaneous) quantity (assigned as 1). Actual spon-
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selective inhibitor NS-398. The attached cell yields (number of cells remaining attached to the tissue culture flask) following NS-398 treat-
ment are expressed as a percentage of control values. IC50 for the reduction of attached cell numbers by NS-398, determined from the dose-response curves, were 82.0 ± 10.1 μM for HT29 and 78.6 ± 11.1 μM for S/KS. Each experiment was carried out with triplicate determinations for each NS-398 dose (20–100 μM), and the data presented are the means of three such experiments; bars, SD. C, dose-dependent induction of apoptosis of HT29 and S/KS carcinoma cells determined after 4 days of treatment with NS-398. The data indicate the quantity of floating cells as a proportion of the total cell number (attached and floating) following NS-398 (20–100 μM) treatment in relation to the respective control (spontaneous) quantity (assigned as 1). Actual sponta-
nous levels of apoptosis were 5.6 ± 3.2% for HT29 and 9.2 ± 2.0 for S/KS. The data presented are from the same experiments as those described in B and are the means of the three experiments; bars, SD. The proportion (mean percentage ± SD) of floating cells that were apoptotic in control and NS-398-treated cultures was as follows: HT29—control, 82.9 ± 3.1%; 20 μM, 87.6 ± 5.3%; 50 μM, 85.0 ± 2.8%; 100 μM, 92.2 ± 2.0%; and S/KS—control, 96.8 ± 3.9%; 20 μM, 92.9 ± 3.1%; 50 μM, 95.6 ± 3.6%; and 100 μM, 94.0 ± 4.2%. These data indicate that the increase in floating cells upon NS-398 treatment was due almost entirely to the induction of apoptosis. The proportion of apoptotic cells in the adherent cell fraction of control cultures was 0.2 ± 0.2% for HT29 and 1.5 ± 1.0% for S/KS (t = 96 h) and did not change significantly following treatment with NS-398.

A. +ve HT29 S/KS +ve COX-2
α-TUBULIN

B. Attached cell yield (% control)

C. Floating cells (fold control value)

Fig. 1 COX-2 protein expression in HT29 and S/KS colon carcinoma cell lines and the effect of the COX-2-selective inhibitor NS-398 on their growth. A, SDS-PAGE Western blot analysis of attached cell lysates of untreated cultures of HT29 and S/KS carcinoma cells showing the expression of COX-2 protein. + ve, COX-2 protein positive control. Equal loading and transfer was shown by repeat probing with anti-α-tubulin. The results presented are from one of the experiments described below (B and C) and are representative of the two additional experiments. B, dose-dependent growth inhibition of HT29 and S/KS carcinoma cell lines determined after 4 days of treatment with the COX-2-selective inhibitor NS-398. The attached cell yields (numbers of cells remaining attached to the tissue culture flask) following NS-398 treatment are expressed as a percentage of control values. IC50 for the reduction of attached cell numbers by NS-398, determined from the dose-response curves, were 82.0 ± 10.1 μM for HT29 and 78.6 ± 11.1 μM for S/KS. Each experiment was carried out with triplicate determinations for each NS-398 dose (20–100 μM), and the data presented are the means of three such experiments; bars, SD. C, dose-dependent induction of apoptosis of HT29 and S/KS carcinoma cells determined after 4 days of treatment with NS-398. The data indicate the quantity of floating cells as a proportion of the total cell number (attached and floating) following NS-398 (20–100 μM) treatment in relation to the respective control (spontaneous) quantity (assigned as 1). Actual spontaneous levels of apoptosis were 5.6 ± 3.2% for HT29 and 9.2 ± 2.0 for S/KS. The data presented are from the same experiments as those described in B and are the means of the three experiments; bars, SD. The proportion (mean percentage ± SD) of floating cells that were apoptotic in control and NS-398-treated cultures was as follows: HT29—control, 82.9 ± 3.1%; 20 μM, 87.6 ± 5.3%; 50 μM, 85.0 ± 2.8%; 100 μM, 92.2 ± 2.0%; and S/KS—control, 96.8 ± 3.9%; 20 μM, 92.9 ± 3.1%; 50 μM, 95.6 ± 3.6%; and 100 μM, 94.0 ± 4.2%. These data indicate that the increase in floating cells upon NS-398 treatment was due almost entirely to the induction of apoptosis. The proportion of apoptotic cells in the adherent cell fraction of control cultures was 0.2 ± 0.2% for HT29 and 1.5 ± 1.0% for S/KS (t = 96 h) and did not change significantly following treatment with NS-398.

NS-398, a COX-2-selective NSAID, Induces Apoptosis in Colon Carcinoma Cells. The traditional NSAIDs sulindac sulfide and piroxicam, which selectively inhibit COX-1 over COX-2, can induce apoptosis in colon carcinoma cells lacking any COX transcripts (12). Also, sulindac sulfone, which lacks COX inhibitory activity, is chemopreventive in rat models of colon carcinogenesis and induces apoptosis in HT29 cells (13, 23). Hence, COX-independent pathways of NSAID-induced apoptosis and chemoprevention are known to exist. If this is considered along with the different chemical properties of the traditional and COX-2-selective NSAIDs (24), then it is uncertain what effect the COX-2-selective NSAIDs will have on colorectal tumor cells, in particular, whether they retain the ability to induce apoptosis. No study has been carried out to address this question.

NS-398 is a NSAID that is highly selective for COX-2 and does not produce the severity of gastrointestinal side effects associated with the "traditional" NSAIDs, which tend to be either selective for COX-1 or to have little specificity (6, 25). To determine a potential mechanism for the recently established chemopreventive properties of COX-2-selective NSAIDs, we determined the effect of NS-398 on HT29 and S/KS, in particular whether it induced apoptosis in these cell lines. S/KS, which does not express COX-2 (see above), was included in the study because COX-2 is not expressed early in colorectal carcinogenesis nor in a subset of colorectal carcinomas. It is, therefore, important to ascertain the effect of COX-2-selective NSAIDs on tumor cells not expressing COX-2 protein as well as on those that do. The effect of NS-398 on cell growth was determined after 4 days of treatment. NS-398 inhibited the growth of each cell line, as indicated by the reduced attached cell yield of treated cultures relative to control cultures (Fig. 1B). Initial studies showed that NS-398 at concentrations as low as 5 μM measurably inhibited the growth of each cell line and induced apoptosis (data not shown). Dose-response experiments showed that these growth-inhibitory effects increased with increasing NS-398 concentration up to the maximum concentration tested (100 μM; Fig. 1B).

We assessed whether the induction of cell death was a means by which NS-398 exerted its growth-inhibitory effect. It
is well established that in the routine culture of colorectal epithelial tumor cells, the majority of cells that detach from the adherent monolayer and float in the medium have undergone apoptosis (see “Materials and Methods”). For each of the HT29 and S/KS cell lines, the dose-dependent decrease in attached cell yield was associated with a dose-dependent increase in the proportion of the cell population that was floating (Fig. 1, B and C). The extent of the induction of cell death at 100 μM NS-398 (the maximum concentration tested) was 3.8 ± 0.5-fold the control value for HT29 and 5.4 ± 1.0-fold the control value for S/KS (t = 96 h). To determine whether the increase in cell death with NS-398 treatment was due to the induction of apoptosis, the floating cells were analyzed by fluorescence microscopy following staining with acridine orange. This showed that, upon treatment with each dose of NS-398, the proportion of floating cells that were apoptotic was similar to and in some cases greater than the control values of 82.9 ± 3.1% for HT29 and 96.8 ± 3.9% for S/KS (see the legend to Fig. 1). Consequently, the increase in the proportion of the cell population that was floating in NS-398-treated cultures was due almost entirely to the induction of apoptosis. The typically apoptotic appearance of control and NS-398-treated cells in the floating cell population of the HT29 cell line is illustrated in Fig. 2. It is notable that HT29 cells lack wild-type p53 and express a mutant p53 protein (26), and S/KS cells express only high levels of a truncated p53 protein (27). This indicates that, like salicylate and the metabolites of sulindac, NS-398 can induce apoptosis independently of wild-type p53 (2, 3, 23). The HT29 and S/KS cell lines are, however, significantly more sensitive to NS-398-induced apoptosis than to salicylate-induced apoptosis. Treatment with 100 μM NS-398 induces apoptosis to the same extent as treating S/KS cells with 2 mM salicylate and HT29 cells with 5 mM salicylate (3).

We also examined whether the induction of cell cycle arrest accounted in part for the growth-inhibitory effect of NS-398. Neither cell line, whether COX-2 expressing or not, exhibited significant changes in the cell cycle phase distribution upon NS-398 treatment at any of the time points or concentrations tested (see “Materials and Methods”; data not shown). Hence, in contrast to NSAIDs that are not selective for COX-2, NS-398 failed to bring about a cell cycle arrest of colon carcinoma cell lines (2, 3).

The data presented here show for the first time that NS-398, a COX-2-selective inhibitor, induces apoptosis in colon carcinoma cells. As indicated by its effect on the S/KS cell line, NS-398 may induce apoptosis in cells lacking detectable COX-2 protein. In fact, these cells and HT29 (which express COX-2) were similarly sensitive to NS-398. Considering the induction of tumor cell apoptosis as a mechanism of chemoprevention, this result suggests that COX-2 selective NSAIDs may indeed be chemopreventive independent of their inhibition of COX-2. This possibility would mean that COX-2-selective NSAIDs are the same as sulindac sulfone in having the ability to induce apoptosis and be chemopreventive by, as yet undetermined, COX-independent mechanisms (13, 23). It would also be consistent with the recent observations that, in the rat colon, nimesulide (an analogue of NS-398) and SC-58635 inhibit the formation of aberrant crypt foci (9, 10), the cells of which are unlikely to express COX-2.

The fact that the HT29 and S/KS cell lines are similarly sensitive to NS-398-induced apoptosis may indicate that specifically inhibiting COX-2 does not induce apoptosis. There is other evidence to indicate this. Tsuji and DuBois (8) have shown that although overexpression of COX-2 in RIE cells confers resistance to butyrate-induced apoptosis, inhibition of the overexpressed COX-2 activity by sulindac sulfide does not induce significant apoptosis. Nevertheless, there is little doubt that COX-2 activity contributes to tumorigenesis, and it must be assumed that its inhibition is part of the mechanism by which COX-2-selective NSAIDs are chemopreventive (8–11). The COX-2-independent antitumor effect of inducing apoptosis (shown here) enhances the potential of COX-2-selective NSAIDs as chemopreventive agents for human colorectal cancer by suggesting that they may also be antineoplastic when COX-2 is not expressed.

In summary, we have shown that a COX-2-selective NSAID induces apoptosis in colorectal tumor cells and that this effect is independent of COX-2 protein expression. The induction of apoptosis provides a possible explanation for the recently observed chemopreventive property of COX-2-selective NSAIDs and further highlights their potential as chemopreventive agents for human colorectal cancer. Moreover, their ability to induce apoptosis may indicate potential as chemotherapeutic agents for colorectal cancer, as suggested for aspirin and sulindac (28).
References


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