Aspirin Toxicity for Human Colonic Tumor Cells Results from Necrosis and Is Accompanied by Cell Cycle Arrest

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ABSTRACT

Chemoprevention of colorectal cancer using aspirin has been demonstrated in rodents and has been suggested by data from epidemiological studies. The mechanism that accounts for this prevention is unknown, but it is thought to relate to an irreversible inhibition of cyclooxygenase and, subsequently, prostaglandin production. The effect of aspirin on the growth of human colonic tumor cells was determined in an effort to gain insight into a possible mechanism of action. In the two cell lines studied, SW 620 and HT-29, we observed a significant dose- and time-dependent increase in aspirin toxicity in a concentration range of 1.25-10 mM. This result was independent of prostaglandin production, because there was no measurable prostaglandin E2 in cell culture medium. As compared with controls, cells in cultures that contained aspirin were not detached, which suggests that the mechanism of cell death was not apoptosis. Flow cytometric analysis revealed an increase in S phase and G2-M populations as well as the number of subdiploid nuclei in cultures treated with high-dose aspirin. Confirmation that cells were undergoing necrosis in response to aspirin was evident from the presence of cells that bound annexin V and accumulated propidium iodide in the absence of a population that bound annexin alone. The results suggest that aspirin induces cell cycle arrest and causes necrosis at high concentrations in vivo, but does not induce apoptosis. Collectively, these two events, necrosis and cell cycle arrest, may contribute to the chemopreventive effect that seems to result from long-term administration of aspirin in vivo.}

INTRODUCTION

Colorectal cancer is highly prevalent in Westernized countries and represents the second most common cause of cancer death in the United States. However, it is probable that alterations in diet and greater use of diagnostics will reduce the mortality rate and eventually make it one of the more preventable cancers (1, 2). It is estimated that adenomas take from 5-10 years to progress to a carcinoma (3). Because the incidence of adenomas increases with age, it is likely that any means that decreases the aberrant adenoma-carcinoma progression will ultimately translate into reduced mortality from colorectal cancer. Among the more promising means of preventing or retarding the development of colorectal cancer is through the use of NSAIDS, principally aspirin and sulindac as well as a number of experimental compounds the most promising of which are COX-2-specific inhibitors.

Evidence that aspirin is chemopreventive for colorectal cancer comes largely from epidemiological studies. With few exceptions, the data from both retrospective and prospective studies have shown a protective effect for aspirin in humans (4). The mechanism by which aspirin inhibits tumorigenesis is unknown but has been related to the irreversible inhibition of COX. Prostaglandin synthesis is greater in colonic tumors than in the surrounding mucosa (5, 6). This relative increase is attributable to the expression of COX-2, which is inducible in response to a variety of cytokines and growth factors, in contrast to COX-1, which is constitutively expressed in the colonic mucosa (7-9). The induction of COX-2 transcription is notable in tumors and rare in adenomas or normal mucosa (10). Recently, a significant decrease in polyp number and size was demonstrated in COX-2 knockout mice relative to controls in mice with an Apc<sup>Δ716</sup> knockout background (11). Down-regulation of COX-2 expression and the specific inhibition of COX-2 activity independently suppress polyp formation and would, therefore, be expected to limit tumorigenesis. The tumor stimulatory product of COX-2 catalysis, the lack of which results in a decrease in both polyp growth and numbers, has not been identified. Although one or more prostaglandins are obvious potential factors influencing polyp growth and size, there is also compelling evidence that aspirin as well as other NSAIDS has a cytostatic and/or apoptotic effect (12-14).

As a means of determining the mechanism by which aspirin inhibits tumorigenesis, we have examined the effect of aspirin on colonic tumor cell proliferation and the extent to which inhibited proliferation relates to a functional decrease in COX activity. The results suggest that aspirin toxicity results from the perturbation of the cell cycle and ultimately causes necrosis, but does not result from induction of apoptosis. Furthermore, aspirin toxicity does not directly correlate with the inhibition of prostaglandin production.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The human colonic tumor cell lines SW 620, HT-29, and HCT-15 (American Type Culture Collection, Manassas, VA) were maintained in RPMI 1640 containing 10% FBS, 2 mM glutamine, and gentamicin (all from Life Technologies, Inc., Grand Island, NY) at 37°C in a humidified atmosphere containing 5% CO₂. Experiments were performed on cells plated at a density of 5 × 10⁴ or 2 × 10⁴/well in 96 well plates or at a density of 5 × 10³/well in 6 well plates. Twenty-four h after plating the cells, aspirin (Sigma, St. Louis, MO) was added and cultures were incubated with continuous exposure for 5-7 days or for 18, 44, or 72 h. In these latter cultures, the medium containing aspirin was removed at the designated time and replaced with fresh medium. Incubation was then continued for a total of 5-7 days after seeding. Aspirin was solubilized in DMSO (Sigma) before addition to the medium. For all experiments, the effect of each aspirin concentration was assessed in 6 replicate wells in each of two independent assays.

Proliferation and Apoptosis Assays. Cell proliferation was assessed for cultures maintained for 5-7 days after the initial plating using MTT (15), and the results are expressed as the % absorbance for treated wells/controls. Apoptosis in response to aspirin was initially assessed on the basis of DNA fragmentation with material isolated using the Puregene DNA isolation kit (Genta Systems, Inc. Minneapolis, MN). DNA (2-5 μg/sample) was electrophoresed in a 1.2% agarose gel in 1X Tris-borate EDTA, and bands were visualized by staining with ethidium bromide. As a positive control, DNA was isolated from SW 620 cells that were maintained for 5 days after confluence or were exposed to 5 ng/ml vinblastine for 24 h. Necrosis/apoptosis was also assessed on the basis of flow cytometric analysis using two different assays: (a) flow cytometry was performed on isolated nuclei recovered from pelleted cells that had been resuspended in PBS containing 0.1% Triton X-100. For these experiments, cells were exposed for 72 h to the aspirin concentrations indicated. After centrifugation, the pellets containing nuclei were incubated for 20 min at 37°C in PBS containing 180 units/ml RNase A. After a final
cells were plated. and HCT-15 (V). Results are from a representative experiment in which cells were plated.

RESULTS

In the initial experiments using conditions of continuous exposure (5–7 days), aspirin was toxic for all three cell lines (Fig. 1). More detailed analysis to determine the mechanism of toxicity was then initiated using the SW 620 and HT-29 lines. In cultures treated with aspirin, there was no increase in the number of floating cells relative to control cultures. To determine the kinetics of aspirin toxicity, cells were exposed to different concentrations of aspirin for varying times (18, 44, or 72 h or 5–7 days) at which point the aspirin-containing medium was replaced and/or the proliferation assay was performed. To further examine the mechanism that accounts for aspirin toxicity, a flow cytometric analysis was used. For isolated nuclei from both cell lines, there was an increase in the percentage of nuclei that contain subdiploid DNA with increasing aspirin dose (Fig. 3 and Table 1). At relatively low concentrations of aspirin (<2.5 mM), the absence of perturbation of the cell cycle correlates with the absence of toxicity. At the higher doses, toxicity is reflected by an increasing abnormality in the cell cycle pattern and the number of nuclei that are subdiploid. Although there was a decrease in the number of cells that are in G0-G1, this decrease is not accounted for by a corresponding increase in the subdiploid population. In fact, with increasing aspirin concentration, there was an increase in the percentage of cells in both the S phase and G2-M.

Flow cytometric analysis using annexin V was used to establish that necrosis and not induction of apoptosis is the mechanism for cell death in response to high-dose aspirin. Cells exposed to aspirin for 3 days bound annexin V and accumulated PI in a dose-related manner, but there was no increase in the number of cells that bound annexin V only (Fig. 4). An additional analysis using annexin V was performed in which cells were exposed to 10 mM aspirin for 4, 21, 45 or 70 h. At none of the time points was there an increase in the number of cells in apoptosis (annexin V staining only) for either of the two cell lines relative to control, untreated cells. An increase in the number of necrotic cells (annexin V- and PI-positive) was not evident in cells exposed to 10 mM aspirin for less than 45 h.

DISCUSSION

Aspirin has attracted considerable attention as one of many compounds that may be of potential benefit in the chemoprevention of cancer. Recent interest is based primarily on results from epidemiological studies that report with very few exceptions a chemopreventive effect (4, 18). Although the data from these studies are compelling, they provide no insight into a possible mechanism by which NSAIDS inhibit tumor formation and/or growth. An obvious potential mechanism is the well-characterized inhibition of COX activity. Data suggest that COX inhibition could limit tumorigenesis or tumor growth by reducing production of mutagens that result from arachidonic acid metabolism (19) or reducing synthesis of immunosuppressive prostaglandins (20), or both. The most convincing data that prostaglandin production is related to inhibition of tumor cell growth are from mice that develop numerous polyps owing to a truncation mutation in the Apc gene, but develop significantly fewer polyps when a COX-2 gene knockout is superimposed or when in the presence of a COX-2 specific inhibitor (11). However, NSAIDS in general have...
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numerous targets other than COX by which they may inhibit tumor growth. For example, inhibition of glycolysis, uncoupling of oxidative phosphorylation, activation inhibition of the transcription factor nuclear factor κB, disruption of a number of signaling pathways and calcium flux as well as inhibition of a large number of other enzymes including cyclic AMP-dependent protein kinase have been described for NSAIDS (21-25). Each of these mechanisms alone or acting in

Fig. 2. Temporal analysis of aspirin toxicity in SW 620 (a) and HT-29 (b) cell lines. Cells plated at $5 \times 10^3$/well were exposed to aspirin-containing medium for 18 h (○), 44 h (□), 72 h (△), or continuously for 5-7 days (★) before replacing the media and/or analyzing the cell survival using the MTT assay. Each point represents the mean ±SE from six data points. Asterisks between data points indicate significant differences ($P \leq 0.05$) between those two points for that aspirin concentration.

Fig. 3. Representative histograms from flow cytometric analysis of SW 620 cells. Cells exposed to (A) medium only; (B) 5 ng/ml vinblastine for 24 h, attached cells; (C) 5 ng/ml vinblastine, detached cells; (D) medium, cells 5 days postconfluent; (E) 10 mM aspirin for 72 h; (F) 5 mM aspirin for 72 h; and (G) 2.5 mM aspirin for 72 h. Abscissa, units of fluorescence intensity. Ordinate, cell numbers. Peaks that correspond to an approximate fluorescence intensity of 380 units are from nuclei in G0-G1. Additional experimental details are provided in the text, and quantitative data are given in Table 1 for both SW 620 and HT-29 cell lines.
concert with decreased prostaglandin synthesis might hypothetically limit tumorigenesis or account for a decrease in tumor cell growth.

Additional results suggest, furthermore, that the chemopreventive effect of NSAIDS cannot be explained on the basis of prostaglandin synthesis inhibition alone. A number of NSAIDS have antiproliferative effects in cells that lack COX activity altogether. In addition, sulindac sulfone (a metabolite of sulindac sulfoxide) lacks COX inhibitory activity but has an antiproliferative effect in vitro and inhibits carcinogen-induced colon carcinogenesis in vivo (26, 27). In the present study, aspirin was toxic for two human colonic cell lines, but there was no COX activity as evidenced by an absence of PGE2 in the medium from cultures of either cell line. To establish that PGE2 is detected in culture medium and that aspirin inhibits prostaglandin synthesis in vitro, we measured PGE2 production in the medium from human colonic biopsy samples incubated for 4 h at 37°C. Consistent with results from an earlier study, PGE2 was present in the medium containing these explants, and its production was inhibited approximately 50% by 1 μM aspirin (28). This inhibitory concentration is 10^{-3} the concentration that is required for aspirin toxicity in established cell lines. HT-29 cells are reported to produce PGE2 and to synthesize both COX-1 and COX-2 transcripts when cells are incubated in the medium supplemented with 10% FBS, but HT-29 cells do not synthesize PGE2 when the cells are maintained in the absence of serum (14). We maintained SW 620 and HT-29 cells in 0.1% FBS and determined that aspirin toxicity for both cell lines was similar to that achieved using 10% FBS (data not shown). If prostaglandin synthesis in HT-29 cells is dependent on serum stimulation as was concluded by Hanif et al. (14), the difference in prostaglandin synthesis for SW 620 and HT-29 cultures in 0.1 versus 10% FBS was not reflected by a difference in cell survival. This result is consistent with the conclusion of others that the antiproliferative effect of aspirin is not due to prostaglandin synthesis inhibition (14, 26, 29). Finally, we have examined aspirin toxicity in a third human colonic tumor cell line, HCT-15, which has been reported not to produce prostaglandins and does not transcribe COX-1 or COX-2 (14). The toxicity profile for this cell line using an aspirin concentration range of 2.5–20 mM under conditions of continuous exposure was almost identical to that of SW 620 and HT-29 for each aspirin concentration. It is apparent from these results that if COX inhibition limits tumorigenesis, the effect is not directly on tumor cells but is mediated by an effector cell.

A number of studies have shown that NSAIDS induce apoptosis in vitro (12–14, 30, 31) and in vivo (32). However, the data regarding aspirin are inconclusive. Shiff et al. (33) examined HT-29 cells for evidence of apoptosis as a result of exposure to one of four different NSAIDS analyzed. Of the four (piroxicam, indo-
methacin, naproxen, and aspirin), aspirin was the sole NSAID that did not induce apoptosis at a dosage that was demonstrably anti-proliferative. Lu et al. (30) also found that aspirin does not induce apoptosis in chicken embryo fibroblasts. In contrast, Elder et al. (13) reported an increase in the number of detached cells from cultured human colorectal adenoma and carcinoma cell line in response to salicylate and concluded that this reflects an induction of apoptosis. We did not observe an increase in the number of detached cells in response to toxic doses of aspirin.

The results from the fluorescence-activated cell-sorting analyses indicate that there is a proportion of cells that develop an apparent subdiploid DNA content in response to aspirin. The results also indicate that at high concentrations of aspirin, there is a decrease in the proportion of cells in G0-G1 and a relative increase in the percentage of cells in S phase and G2-M. This result is in contrast to data obtained using NSAIDS (13, 22, 31), including aspirin (33), which indicate that the exposure causes the arrest of cells in G0-G1. The data from the present study suggest that aspirin directly or indirectly retards cells from progressing through the S phase and G2-M as rapidly as is normal, which results in a relative accumulation of cells in these two phases. Nevertheless, the antiproliferative effect would be similar to that achieved by placing a block on G0-G1. Coincident with this effect is an increase in the number of cells with subdiploid DNA content that represent dying cells. The absence of a population of cells that bind annexin V but exclude propidium at any time for any toxic aspirin concentration indicates that necrosis and not apoptosis is the mechanism that accounts for aspirin toxicity in these cells. These two events—induction of necrosis and disruption of the normal cell cycle pattern—may be independent events but nevertheless are correlated with toxicity. In theory, high-dose aspirin may indirectly result in damage that induces cell cycle arrest. If unrepaired, the damage may ultimately result in cell necrosis. Although certainly less potent, this effect would be similar in appearance to that which occurs as a result of radiation, which also causes a block at G2-M and eventually necrosis. Vinblastine treatment also resulted in an accumulation of cells in G2-M, which was particularly evident in attached cells, but this is attributed to the well-characterized binding of Vinca alkaloids to microtubules.

The cancer chemopreventive effect of aspirin seems to be achieved only as a result of prolonged intake. The aspirin concentration required for toxicity of tumor cells in vivo exceeds which can be sustained in vivo. However, the extent to which cumulative aspirin toxicity and growth inhibition contributes to a significant decrease in cancer incidence and mortality is yet to be determined.

REFERENCES

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