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Effect of Aspirin on Induction of Apoptosis in HT-29 Human Colon Adenocarcinoma Cells

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ABSTRACT. Aspirin (ASA) and other nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit colorectal tumorigenesis. Apoptosis is a critical determinant of tissue mass homeostasis and may play a role in carcinogenesis. We studied the effect of ASA on the survival of a human colon cancer cell line using more sensitive methods than we had applied previously. ASA induced apoptosis in HT-29 colon adenocarcinoma cells at concentrations ≥1 mM as established by: (a) morphological changes consistent with apoptosis in cells examined by fluorescence microscopy and semi-thin cell sections, and (b) DNA strand breaks: 45% of the cells were TdT-mediated dUTP nick end labeling (TUNEL) positive at 3 mM at 72 hr, and 70% were positive by the comet assay. Electron microscopy also confirmed the induction of apoptosis by ASA. ASA-induced apoptosis was not associated with: (a) a ladder pattern on genomic DNA electrophoresis, or (b) a subdiploid peak on flow cytometry. Apoptotic bodies were virtually absent on standard morphological assessments and only a few were detected on semi-thin sections. For the above reasons, this apoptosis induced by ASA is "atypical," and the unusual features of ASA-induced apoptosis, besides their taxonomic value, may offer clues to the mechanisms that control the process of apoptosis or perhaps the cancer chemopreventive properties of this compound. These findings demonstrate that ASA induces apoptosis in human colon cancer cells, bolstering the hypothesis that apoptosis may be a mechanism by which NSAIDs inhibit colon carcinogenesis. These findings should be examined in animal and/or clinical research studies *in vivo*. BIOCHEM PHARMACOL **55**;1:53–64, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. aspirin; NSAIDs; apoptosis; DNA strand breaks; chemoprevention

NSAIDs¶ inhibit colorectal tumorigenesis. Abundant data from epidemiological studies have shown that several NSAIDs decrease the incidence of, and mortality from, colon cancer [reviewed in Ref. 1]. Animal studies, which actually preceded epidemiological observations, showed that NSAIDs, including ASA [2] and indomethacin [3], reduce the number and size of carcinogen-induced colon tumors in rodents. Indomethacin and sulindac also decrease the number and size of colon adenomas in patients with familial adenomatous polyposis [4, 5]. It is widely assumed that the anti-neoplastic effect of NSAIDs could involve inhibition of PG synthesis [6, 7], a well-established target for NSAID action. However, COX-independent mechanisms are being considered [8].

Our previous experiments demonstrated that several NSAIDs inhibit proliferation and induce apoptosis in HT-29 and HCT-15 colon adenocarcinoma cells [8–10]. We found that, in HT-29 cells, ASA reduced the proliferation rate, altered the morphology, and increased the proportion of cells in the G_0/G_1 phase of the cell cycle while reducing those in S phase. However, ASA at concentrations between 400 and 1500 μM never induced a subdiploid peak in DNA content on FACS analysis or a ladder pattern on electrophoresis of genomic DNA, nor produced apoptotic bodies. Therefore, we concluded that ASA did not induce apoptosis in these cells [10].

Two reasons why apoptosis may not have been detected after ASA treatment in our initial studies could be that: (a) the concentrations we employed were too low; and/or (b) our methodology was not sensitive enough to detect ASA-induced apoptosis. Therefore, to determine whether ASA could induce apoptosis in colon cells, we studied the effect of an expanded range of ASA concentrations and used sensitive methods for apoptosis detection.

ASA is the most frequently studied NSAID colon cancer chemopreventive agent in the population at average-risk for the development of this tumor [1]. Therefore, it is of great importance, from a mechanistic and therapeutic standpoint, to ascertain whether ASA, like virtually all

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¶ *Abbreviations:* NSAIDs, nonsteroidal anti-inflammatory drugs; ASA, aspirin; PG, prostaglandin; FACS, fluorescence-activated cell sorting; FITC, fluorescein-5-isothiocyanate; COX, cyclooxygenase; TUNEL, TdT-mediated dUTP nick end labeling; TdT, terminal deoxynucleotidyl transferase; HBSS, Hanks' buffered salt solution; DAPI, 4,6-diamidino-2-phenylindole; PI, propidium iodide; and EM, electron microscopy.

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other NSAIDs, induces apoptosis in colon cancer cells *in vitro*. Such information is important to any hypothesis concerning the effect of ASA and this family of compounds on the colon *in vivo*.

Apoptosis, a genetically directed active process of cellular self-destruction, which contributes to the regulation of cell mass in many tissues [11, 12], most likely plays a key role in the tissue mass homeostasis of the colonic mucosa. The cardinal features of apoptosis include cell shrinkage, condensation of cytoplasm and chromatin, nuclear fragmentation, and formation of cell surface "blebs." The nuclear collapse is associated with extensive damage to chromatin and DNA-cleavage into oligonucleosomal-length DNA fragments after activation of a calcium-dependent endogenous endonuclease [13]. Apoptosis-associated DNA degradation can also be recognized as a large number of DNA strand breaks *in situ* [14]. Necrosis, on the other hand, is a passive, non-genetically regulated process, often caused by an intense external stimulus. Cellular injury leads to the influx of water and ions, resulting in mitochondrial edema (and eventually rupture), flocculation of chromatin, and early rupture of the plasma membrane [15]. In addition to these two clearly distinguishable types of cell death, there are intermediate forms, which either show only some features of apoptosis or share features of both necrosis and apoptosis [16].

The detection of apoptosis is based on techniques that monitor either characteristic biochemical (e.g., nicking or fragmentation of DNA) or morphological (e.g., cell morphology, status of cellular organelles or membranes) features of these processes [16–18]. In our present studies, we have employed an array of methods for the detailed study of the effect of ASA on the survival of colon cancer cells. This paper summarizes our findings.

MATERIALS AND METHODS

Cell Lines

Human colon adenocarcinoma cell lines (HT-29) were obtained from the American Type Culture Collection (ATCC, Rockville, MD), and grown as monolayers, according to the instructions of the ATCC. Cells were grown in McCoy's 5A (GIBCO, Grand Island, NY) medium and supplemented with 10% fetal bovine serum (Gemini Bio-Products, Calabasas, CA), streptomycin (10,000 U/mL), and penicillin (10,000 U/mL). Cells were incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Cells were plated at a density of 1×10^6 cells/100 cm^2 culture dish (Falcon, Becton-Dickinson, NJ). Proliferation was determined by counting an aliquot of trypan blue-stained cells with a hemacytometer as described [9].

Reagents

ASA (Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and stored at −20°; the concentration of DMSO was equalized in all dishes. Cells were harvested by using 0.05% trypsin/EDTA (GIBCO) and then resuspended in 1% BSA in PBS (PBS/BSA).

Bivariate Flow Cytometry: TUNEL and PI Staining

Cells (2×10^6) were washed with PBS/BSA and fixed in 200 μL of 2% paraformaldehyde (pH 7.4) in PBS for 15 min at 4°. Cells were washed with PBS/BSA and incubated with 0.1% Triton-X 100 in PBS for 5 min on ice. A one-step procedure to detect DNA strand breaks (TUNEL) was employed [19, 20], using a commercial kit (Boehringer–Mannheim, Indianapolis, IN); DNA strand breaks *in situ* indicate apoptotic cells. The "Enzyme Solution" contained TdT. The "Label Solution" contained the labeled nucleotide in reaction buffer [3 nM FITC-dUTP, 2 mL 25 mM $CoCl_2$ and TdT buffer (30 mM Tris, pH 7.2, 140 mM sodium cacodylate)]. The cells were washed with PBS and resuspended in 50 μL/tube of TUNEL Reaction Mixture" (consisting of 50 μL of Enzyme Solution plus 450 μL of Label Solution). Omission of TdT from the staining protocol constituted the negative control. Cells were incubated for 60 min at 37° in the dark in a humidified atmosphere, washed again with PBS/BSA, resuspended in PBS with 10 μg/mL PI (Sigma) and 200 μg/mL RNase A and incubated at room temperature for 15 min prior to flow cytometric measurement. Apoptotic cells having DNA strand breaks incorporate FITC-conjugated dUTP and emit fluorescence on flow cytometric analysis.

The fluorescence of cells stained with PI and FITC-conjugated dUTP (TUNEL reaction) was measured using a Coulter-XL flow cytometer (Coulter, Miami, FL). PI and FITC fluorescence signals were collected in linear and log scale, respectively. The cell TUNEL fluorescence level was defined as events giving a signal stronger than 2% above that of the corresponding negative control. Parameters [linear forward scatter (FSC), log side scatter (SSC), log of FITC, and linear of PI] were collected in listmode files. All data were further analyzed on a Profile Elite Work station (Coulter).

TUNEL Assay on Cells Fixed on Microscope Slides

About 50,000 freshly harvested cells were distributed on glass slides by centrifugation at $89.4 \times g$ in a cytospin well (Cytospin 2, Shandon Inc., Pittsburgh, PA) for 5 min and fixed in 2% paraformaldehyde in PBS (pH 7.4) for 15 min. Following washing in PBS, they were treated with 0.1% Triton X-100 in PBS for 5 min and washed with PBS. Then, 25 μL of TUNEL buffer from the Boehringer–Mannheim commercial kit was added onto the slides, which were incubated in a humidified chamber for 60 min at 37°. Following washes, the specimens were mounted and sealed with nail polish. The cells were examined by fluorescence microscopy.

DAPI-Sulforhodamine Staining

Cells were evaluated for apoptosis by studying their morphology after staining with DAPI, which highlights cellular DNA. About 50,000 freshly harvested cells were distributed on glass slides as described above and fixed by immersion in acetic acid:ethanol (1:9, v/v) for at least 20 min. Upon drying, the slides were treated with HBSS, and stained with 1 μg/mL DAPI-10 μg/mL Sulforhodamine (Sigma) in 1,4-piperazinediethanesulfonic acid (PIPES) buffer [21]. Following several washes with HBSS, the specimens were mounted and sealed with nail polish. The cells were examined by fluorescence microscopy. The morphological criteria of apoptosis included: (a) cytoplasmic and nuclear shrinkage; (b) chromatin condensation; and (c) cytoplasmic blebbing with maintenance of the integrity of the cell membrane (zeiosis) [22].

Comet Analysis

Cells were treated and harvested as described previously [23, 24]. Each frosted slide (Dakin, VWR Scientific) was covered with 100 μL of 0.5% normal melting point agarose in PBS (Ca^{2+}, Mg^{2+} free) at 45°, immediately covered with a cover slip, and then kept at 4° for 10 min to allow the agarose to solidify. About 200,000 treated or control cells were suspended in 10 μL of PBS and mixed with 75 μL of 0.5% low melting point agarose at 37°. After gently removing the cover slip, the cell suspension was pipetted rapidly onto the first agarose layer, spread smoothly using a cover slip, and kept at 4° for 10 min. After removal of the cover slip, a third layer of 0.5% low melting agarose (75 μL) at 37° was added, spread using a cover slip, and allowed to solidify at 4°. After removing the cover slip, the slides were immersed in freshly prepared cold lysing buffer (2.5 mM NaCl, 100 mM Na_2EDTA, 10 mM Tris, pH 10, with 1% Triton X-100 and 10% DMSO added just before use) overnight at 4°. The slides were removed from the lysing solution, drained, and then placed side-by-side in a horizontal gel electrophoresis tank, avoiding spaces, and with the agarose end facing the anode. The tank was filled with fresh electrophoresis buffer (1 mM Na_2EDTA and 300 mM NaOH) to a level approximately 0.25 cm above the slides. The slides were left in the high pH buffer for 20 min, followed by electrophoresis, conducted at room temperature for 20 min at 25 V adjusted to 300 mA by raising or lowering the buffer level in the tank. After electrophoresis, the slides were gently washed by placing them in a tray and flooding them slowly with three changes of 0.4 M Tris, pH 7.5, each for 5 min. After neutralization, the slides were stained with 50–100 μL of 20 μg/mL ethidium bromide in distilled H_2O and covered with a cover slip. The slides were examined using a fluorescence microscope with filters of 515–560 nm from a 100 W mercury lamp and a barrier filter of 590 nm, and photographed (Labophot, Nikon Inc., Melville, NY). Presence of the characteristic tail was the criterion to identify cells with damaged DNA. The percentage of cells with damaged DNA among all of the cells present on the slide was determined by counting ten randomly selected high-power fields.

DNA Degradation

Genomic DNA was obtained from 0.5×10^6 HT-29 cells by lysis in 0.04 M Tris–acetate/1 mM EDTA/0.25% NP-40. These extracts were digested with 100 μg/mL boiled RNase IIA (Sigma) followed by treatment with 1 mg/mL proteinase K (Boehringer–Mannheim). Aliquots of DNA from the equivalent of 2×10^5 cells were mixed with sample buffer (final concentration: 0.025% bromophenol blue, 3.0% glycerol) and resolved on 1.8% agarose gels. DNA was visualized by UV transillumination and photographed using Polaroid 667 film.

Transmission EM

Control HT-29 cells or those treated with ASA or sulindac sulfide for 72 hr and that remained attached to the surface of the culture dishes were gently washed with serum-free medium, and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). These cells were scraped from the surface of the dishes and pelleted by spinning for 5 min at $10,000 \times g$. The cells were osmicated with 1% osmium tetroxide, then block stained, dehydrated in graded ethanol, infiltrated with propylene oxide, and embedded with EMBED (Electron Microscopy Science, Fort Washington, PA) overnight and cured in a 60° oven for 48 hr. Silver sections were cut with an Ultracut E (Reichert–Jung) microtome, collected on a formvar and carbon-coated grid (Electron Microscopy Science), stained with uranyl acetate and Reynolds' lead citrate, and viewed on a JEOL 100 CX II electron microscope.

Light Microscopy of Semi-Thin Sections of Cells

HT-29 cells treated with ASA or sulindac sulfide for 72 hr were divided into two fractions: (1) those remaining adherent to the culture dish, and (2) those that detached from the surface and were floating in the culture medium. Both fractions and only the adherent fraction from control HT-29 cells were gently washed with serum-free medium and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4). The adherent cells were scraped from the surface of the dishes, and both fractions were pelleted separately by spinning at $10,000 \times g$ for 5 min. Then the cells from each fraction were osmicated with 1% osmium tetroxide, block stained, dehydrated in graded ethanol, infiltrated with propylene oxide, embedded with EMBED overnight, and cured in a 60° oven for 48 hr. Semi-thin sections, 0.9 μm in thickness, were cut with an Ultracut E microtome, placed on microscope slides, heated to dryness on a hot plate, stained with 0.05% methylene blue in 0.05% sodium borate and 0.05% Azure II, mounted with coverslips, and examined by light microscopy (Op-

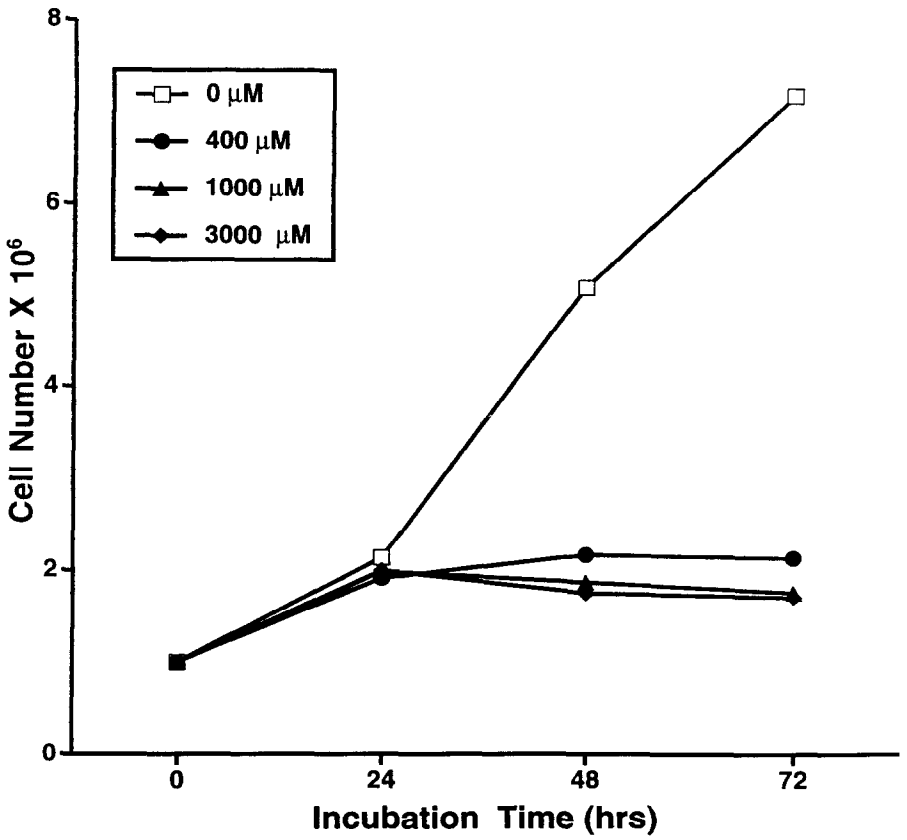

FIG. 1. Proliferation of HT-29 cells treated with aspirin. Cells were treated with ASA at the concentrations shown in the inset, as described in Materials and Methods. Each number represents the average of triplicate plates.

tiphot, Nikon, Inc.). The cells with morphological features of apoptosis were quantified by counting the number of apoptotic cells observed relative to 300 randomly distributed normal cells.

RESULTS

Effect of Aspirin on Proliferation and Cell Cycle Distribution of HT-29 Cells

HT-29 cells were plated at a density of 2×10^6 cells/100-cm^2 tissue culture dish, with or without ASA supplemented to the culture medium. Cells were harvested 24, 48, 72, or 96 hr after plating. In this and all subsequent studies, unless otherwise indicated, we evaluated the cells attached to the surface of the culture dish at the time of harvesting combined with the detached cells floating in the culture medium.

In agreement with our previous observation [10], ASA profoundly reduced the proliferation rate of HT-29 cells (Fig. 1). This effect was apparent 48 hr after the addition of ASA to the culture medium. The inhibition by ASA was concentration- and time-dependent, being maximal at 3 mM, the highest concentration studied. At 72 hr, ASA decreased the number of cells by 70% at 400 μM, 75% at 1 mM, and 76% at 3 mM, compared with controls.

Table 1 and Fig. 2 indicate the effects of ASA on the cell cycle distribution of HT-29 cells. ASA at 400 μM induced limited or no change in the cell cycle distribution of cells. At concentrations of 1 and 3 mM, ASA increased the

TABLE 1. Effect of aspirin on cell cycle distribution of HT-29 cells

	% G_0/G_1			% S			% G_2/M		
ASA (μM)	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr	24 hr	48 hr	72 hr
0	48	49	52	32	35	30	20	16	18
400	49	51	57	32	33	26	21	16	17
1000	58	69	73	15	9	7	23	22	20
3000	77	81	82	7	5	5	16	14	13

HT-29 cells were treated with ASA and harvested at 24, 48, or 72 hr. The percentage of cells in each phase was determined by flow cytometry as described in "Materials and Methods." This protocol was followed three times, and values each time were within 5%.

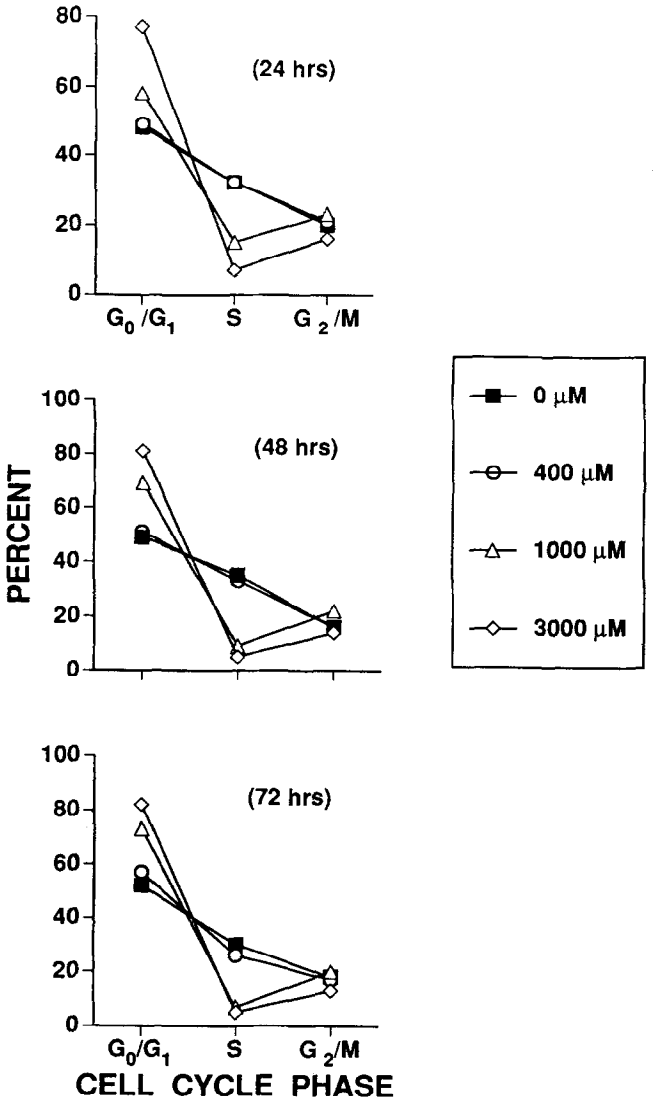

FIG. 2. Cell cycle distribution of HT-29 cells treated with aspirin. HT-29 cells were treated for 24, 48, or 72 hr with various concentrations of ASA. Their cell cycle phase distribution was determined by flow cytometry, as described in "Materials and Methods."

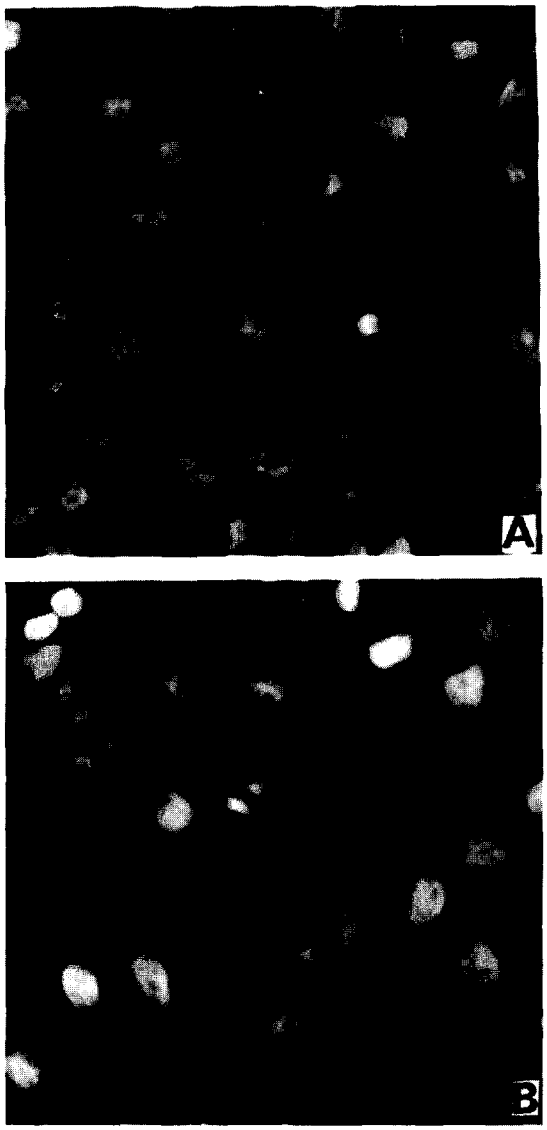

FIG. 3. Fluorescence photomicrographs of HT-29 cells treated with aspirin. HT-29 cells were treated with control medium (A) or 3 mM ASA (B) for 72 hr and stained with DAPI, as described in Materials and Methods. Several ASA-treated cells show irregular, condensed nuclei, with increased fluorescence intensity, suggestive of apoptosis. Apoptotic bodies are absent; the two adjacent cells near the center that show attached minuscule fragments represent a rare example of changes reminiscent of apoptotic bodies. Original magnification: 40×.

proportion of cells with G_0/G_1 phase DNA content and decreased those with S phase DNA content; there was no change in the proportion of cells with G_2/M DNA content. The effect of ASA treatment on the cell cycle phase distribution was noted as early as 24 hr, was maximal at 48 hr, persisted throughout the 72-hr experimental period, and was concentration-dependent. At 48 hr, 3 mM ASA increased the proportion of cells in G_0/G_1 by 65% and decreased those in S phase by 86%.

Effect of Aspirin on Induction of Apoptosis in HT-29 Cells

ASA treatment of HT-29 cells altered their morphology and induced DNA strand breaks in a manner consistent with apoptosis. That the changes were indeed induced by apoptosis and not necrosis was confirmed by EM.

(1) MORPHOLOGICAL CHANGES. The morphology of ASA-treated HT-29 cells was monitored by fluorescence microscopy after staining with DAPI. ASA induced significant morphological changes that became evident 24 hr after treatment with 3 mM ASA and were maximal in cells treated for 72 hr. ASA-treated cells had irregular, condensed nuclei with increased fluorescence intensity (Fig. 3). However, nuclear fragmentation, which is characteristically associated with apoptosis, and clearly identifiable apoptotic

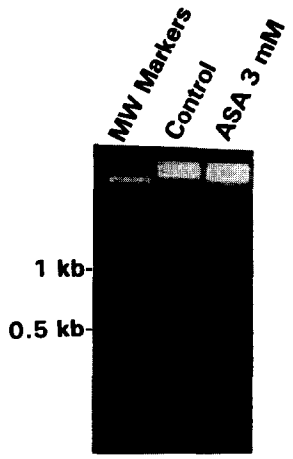

FIG. 4. Electrophoresis of genomic DNA from HT-29 cells treated with aspirin. Genomic DNA, isolated from control HT-29 cells and those treated for 72 hr with 3 mM ASA was fractionated on 1.8% agarose gels as described in "Materials and Methods." The DNA in each lane corresponds to 2×10^5 cells. The positions of DNA molecular weight standards are shown on the left. No fragmentation of DNA in response to ASA is apparent.

bodies were rarely, if ever, seen. The cytoplasm of the cells always appeared demarcated by an intact plasma membrane. Treatment with as much as 5 mM ASA for 72 hr failed to produce more pronounced morphological changes. Longer incubations (i.e. up to 96 hr) also failed to capture the cells in the classic late morphologic manifestations of apoptosis.

(II) CHANGES IN DNA INTEGRITY. ASA induced endonucleolytic activity in the HT-29 cells, but, as with the morphological changes, this was to a lesser degree than is customarily associated with fully developed apoptosis. Several different methods were implemented to assess genomic DNA integrity of ASA-treated cells:

(i) Size fractionation of genomic DNA/DNA ladder formation. HT-29 cells treated with 3 mM ASA showed no evidence of a ladder pattern on agarose gel electrophoresis of their genomic DNA (Fig. 4). As a control, we documented that the cells used to extract the genomic DNA for this experiment were derived from those that responded to ASA with the expected antiproliferative effect. DNA extracted from cells treated with ASA concentrations below 3 mM also did not show a ladder pattern (data not shown), as was observed previously [10].

TABLE 2. Percentage of TUNEL-positive HT-29 cells after treatment with aspirin; time and concentration response

ASA (μM)	% TUNEL-positive cells		
	24 hr	48 hr	72 hr
0	1.3 ± 0.5	1.9 ± 0.3	1.5 ± 0.2
400	2 ± 0.3	1.8 ± 0.5	5 ± 0.3
1000	3.5 ± 0.6	3.7 ± 0.4	14.2 ± 1.6
3000	3.5 ± 0.2	14.3 ± 0.9	45.3 ± 17.3

Values are means ± SD, N = 4.

(ii) Flow cytometric analysis of DNA content. Flow cytometric assessment of the DNA content of ASA-treated HT-29 cells failed to show a subdiploid (apoptotic) peak (Fig. 5, inset). This was true of cells treated with ASA at all concentrations and all treatment durations examined, including treatment with 5 mM ASA for 72 hr.

(iii) Flow cytometric analysis of DNA strand breaks. Flow cytometry was applied to control or ASA-treated HT-29 cells stained by: (a) the TUNEL method alone; or (b) the TUNEL method and PI. The TUNEL method, which detects free 3′-OH groups at DNA strand breaks, is more sensitive in ascertaining nucleolytic activity than flow cytometric analysis of DNA content exclusively by PI staining.

As is shown in Table 2, the proportion of ASA-treated cells that exhibited DNA strand breaks as assessed by TUNEL staining increased in a time- and concentration-dependent manner; nearly half the cells were TUNEL-positive (i.e. were stained by the TUNEL technique) when incubated with 3 mM ASA for 72 hr. Of note, when cells were treated with 3 mM ASA for 96 hr, 78% of them were TUNEL-positive (data not shown). In contrast, the pro-

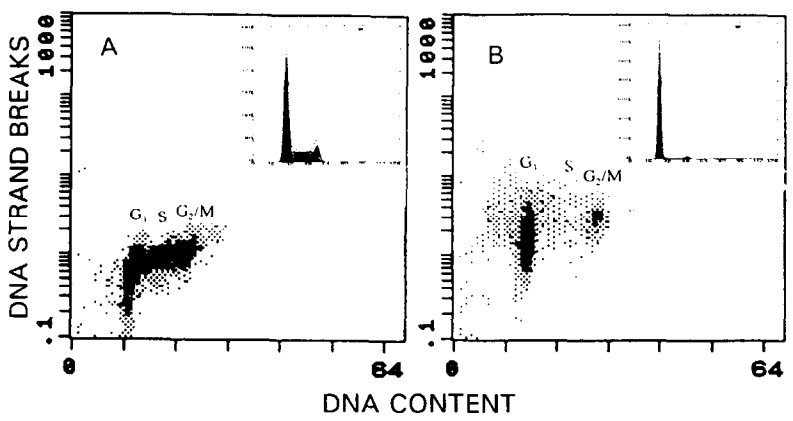

FIG. 5. Flow cytometric determination of DNA content and DNA strand breaks in HT-29 cells treated with aspirin. DNA strand breaks (TUNEL positivity) occurred in cells treated with 3 mM ASA for 72 hr (panel B), but not in controls (panel A). DNA strand breaks occurred in all phases of the cell cycle, as indicated by TUNEL-positive cells with all possible DNA contents. The DNA histogram (inset), which represents all cells, showed no subdiploid peak in response to ASA treatment (B).

portion of TUNEL-positive control cells remained essentially unchanged during the period of observation (≤2.0%). Negative control staining reactions, done with TdT omitted from the protocol, exhibited no positive staining signals.

Flow cytometric analysis of cells labeled simultaneously by TUNEL and PI staining made it possible to quantify the incorporation of dUTP relative to their cellular DNA content (i.e. relative to their position in the cell cycle). Thus, we evaluated whether ASA-treated cells were induced preferentially into apoptosis in a particular phase of the cell cycle. As demonstrated in Fig. 5, TUNEL-positive cells were distributed equally amongst the G_0/G_1, S, and G_2/M cell cycle phases. Therefore, ASA-induced apoptosis occurred regardless of the location of the cell in the cell cycle.

(iv) Detection of DNA strand breaks by fluorescence microscopy. To evaluate the occurrence of DNA strand breaks relative to morphological features of apoptosis, we applied the TUNEL method to control or ASA-treated HT-29 cells fixed to microscope slides. Examination of up to ten high power fields per sample exhibited the following:

(a) The percentage of apoptotic (i.e. TUNEL-positive) control or ASA-treated cells closely matched the proportion detected by flow cytometric analysis of TUNEL-stained control or ASA-treated HT-29 colon cancer cells (Fig. 6); on average about 50% of the cells were TUNEL-positive after 72 hr of treatment with 3 mM ASA.

(b) The morphological features of the ASA-treated, TUNEL-positive cells did not exhibit the classical characteristics of fully developed apoptosis [22]. Notably, the distribution of the fluorescent label was uniform throughout the nuclei. More importantly, we never observed fragmented nuclei or apoptotic bodies indicative of late stage apoptosis, even after prolonged incubation with high concentrations of ASA. In contrast, when these cells were treated with other NSAIDs, such as sulindac sulfide, they clearly exhibited the hallmark morphological features of classical apoptosis, such as nuclear fragmentation and apoptotic body formation [9]. Similar findings were obtained when ASA-treated cells were stained with DAPI.

(c) Inspection of the cytoplasm, albeit limited in nature, revealed that the TUNEL-positive cells had intact cytoplasmic membranes, a requisite characteristic of apoptotic cells until very late in the evolution of this process.

(v) Comet assay. To further assess DNA strand breaks induced by ASA, we evaluated control HT-29 cells or those treated with different concentrations of ASA for 72 hr by the comet assay [22, 23]. This technique involves performing gel electrophoresis on single cells and can detect single-strand DNA breaks by their characteristic migration pattern in a specially prepared electric field.

Less than 1% of the control cells showed evidence of DNA damage in the form of the typical tail formation [25]. In contrast, cells treated with ASA exhibited a concentration-dependent increase in DNA damage. DNA damage was evident in 5.1% of cells treated with 400 μM ASA, 25% of those treated with 1 mM ASA, and 72% of cells treated with 3 mM ASA for 72 hr (Fig. 7). As shown in Fig. 8, compared with the TUNEL method, this technique revealed a consistently higher percentage of cells with DNA damage; the number of cells displaying DNA damage by the comet assay was increased up to 2-fold of the amount detected by TUNEL staining.

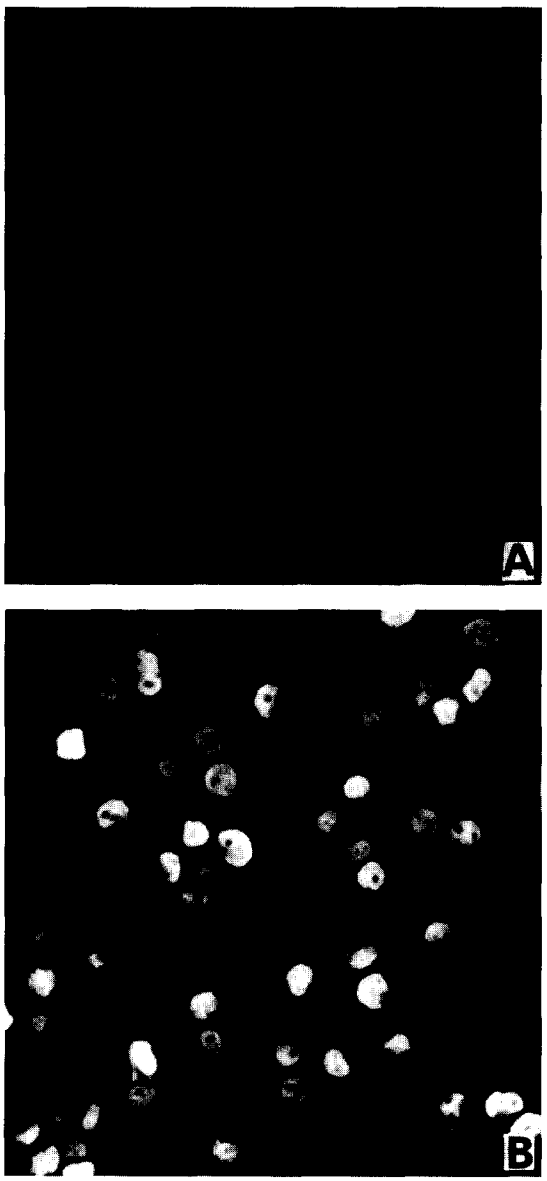

FIG. 6. Detection of DNA strand breaks by fluorescence microscopy (TUNEL) in aspirin-treated HT-29 cells. Cells fixed on a microscope slide were assayed for DNA strand breaks by the TUNEL method. In contrast to control cells (panel A), cells treated with 3 mM ASA for 72 hr (panel B) showed fluorescent labeling of their nuclei, indicating DNA strand breaks. TUNEL-positive cells had intact cytoplasm.

(vi) Morphologic study of HT-29 cells by EM. Both the TUNEL and comet methods are highly sensitive for the

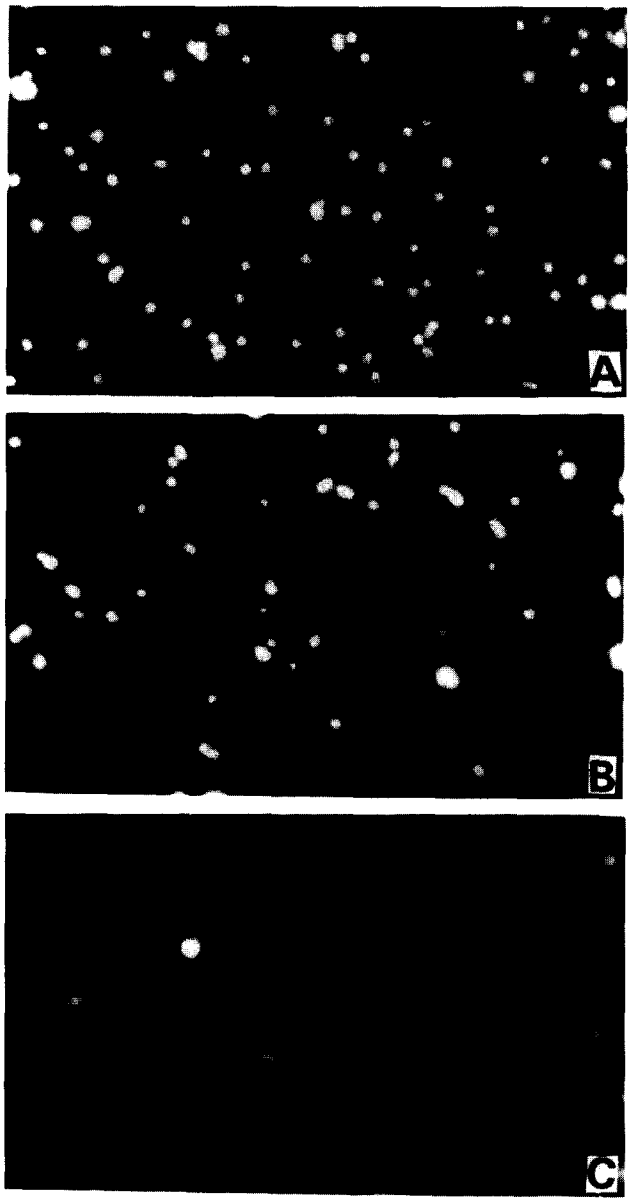

FIG. 7. Aspirin-induced DNA damage in HT-29 cells detected by the comet assay. Cells were suspended in agarose to make a gel, were mounted on a microscopic slide, lysed, electrophoresed, stained with a dye that binds DNA, and viewed with a fluorescence microscope. DNA containing breaks moved from the brightly fluorescent core (the head) towards the anode, forming the image described as a *comet*. Control cells (panel A) showed no comet formation, which appeared in cells treated with 1 mM ASA for 72 hr (panel B), and became almost universal in cells treated with 3 mM ASA for 72 hr (panel C).

detection of DNA damage associated with apoptosis [18, 25]. They are, however, not completely specific for apoptosis-induced DNA damage [26]. Other forms of DNA damage, such as that generated by necrosis, can also produce positive signals in these assays. Therefore, it was critical to verify that apoptosis (and not necrosis) was occurring in ASA-treated cells. To accomplish this, control HT-29 cells or those treated for 72 hr with 1 or 3 mM ASA were evaluated by transmission EM. For technical reasons, we were able to examine by EM only the cells attached to the culture dish.

As depicted in Fig. 9, ASA (1 and 3 mM) induced the ultrastructural changes typical of apoptosis by 72 hr. The nuclear chromatin was compacted, with finely granular masses marginated against the nuclear envelope; the cytoplasm was condensed; the nuclear (and cellular, in the case of the 1 mM ASA example in this figure) outline was convoluted; and the ultrastructural features of the cytoplasmic organelles were preserved.

Examination of several fields disclosed that 1 mM ASA (Fig. 9B) induced a very low frequency of apoptosis and no necrosis in the adherent cell fraction. A 3 mM concentration of ASA (Fig. 9C) induced morphological features consistent with apoptosis, but few lysed, necrotic cells were also observed. Again, as noted in the other assays, clear evidence of late stage apoptosis was not seen in the adherent cell fraction by EM.

(vii) Morphologic study of semi-thin sections of HT-29 cells. As indicated above, for technical reasons we were unable to examine the floating cells that were detached from the culture dishes by EM. Nevertheless, we evaluated them at high resolution by examining semi-thin (0.9 μm) sections of control or ASA-treated HT-29 cells (Fig. 10). Although this method is not as definitive as EM, because it cannot reach its fine level of resolution, it nonetheless provides sufficient detail to allow confident assessment of the integrity of the cells.

In this study, we examined control HT-29 cells or those treated with ASA at 1 or 3 mM for 72 hr. The attached and detached cell fractions were fixed and embedded exactly as for the EM study, cut into semi-thin sections, placed on microscope slides, and examined separately with a light microscope. The adherent cell fraction of control cells shows sheets of polygonal cells with completely intact nuclei and cytoplasms (a representative area is depicted in Fig. 10A). No nuclear or cytoplasmic condensation was noted in any of the cells. HT-29 cells treated with 1 mM ASA and which adhered to the culture dish appeared identical to the control cells (Fig. 10B). There were very few cells treated with this concentration of ASA that exhibited features of apoptosis, which agrees with the evaluation by EM (Fig. 9). However, in the adherent fraction of cells treated with 3 mM ASA, about 6% of the cells exhibited features characteristic of apoptosis (Fig. 10D). Thus again, with this assay ASA clearly induces apoptosis in HT-29 cells in a concentration-dependent manner.

As expected, the floating fraction of cells was enriched in dead and dying cells. Control plates did not produce enough floating cells that could be harvested by our method. About 9% of the floating cells from the 1 mM ASA-treated plates showed features typical for apoptosis (Fig. 10C). Of note, some of the cells exhibited characteristics of late stage apoptosis with nuclear fragmentation and apoptotic body formation. This was the only method that

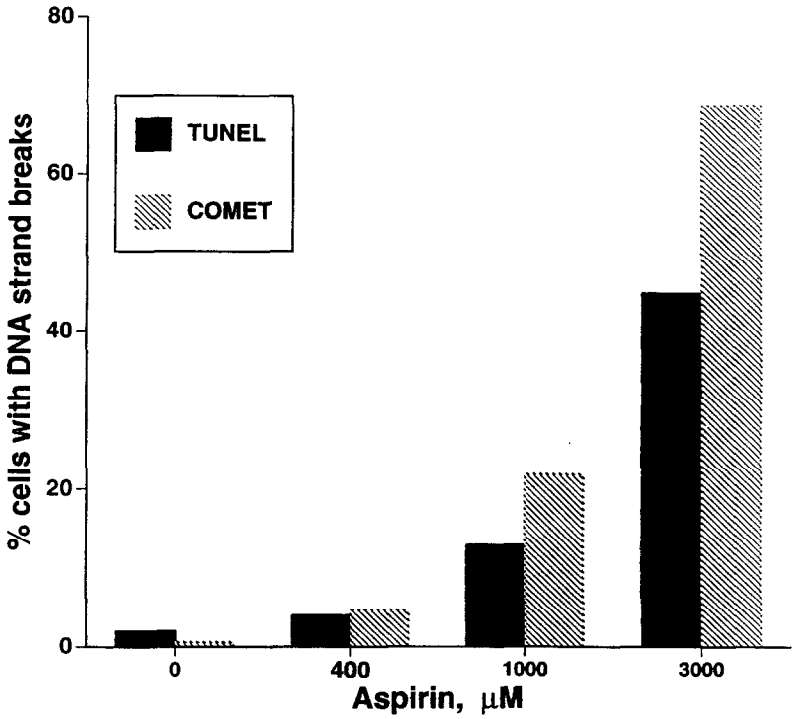

FIG. 8. Aspirin-induced DNA strand breaks detected by TUNEL or comet assay. HT-29 cells were treated with several concentrations of ASA for 72 hr. Compared with the TUNEL method, the comet assay revealed a consistently higher percentage of cells with DNA damage. Each percentage represents the average of three experiments.

we implemented that could capture ASA-treated HT-29 cells in a late stage of apoptosis. The floating fraction from cells treated with 3 mM ASA showed an even greater fraction of apoptotic cells (Fig. 10E), around 20%. However, it should be noted that some cells of the floating fraction that were treated with either concentration of ASA also showed some morphological features of necrosis, such as vacuolization of the cytoplasm. Nevertheless, the examination of these semi-thin sections clearly confirms that ASA induces apoptosis in HT-29 colon adenocarcinoma cells.

DISCUSSION

Our data demonstrate that ASA exerts two profound effects on the HT-29 colon adenocarcinoma cells: (a) it inhibits their proliferation, and (b) it reduces their survival by inducing cell death by apoptosis. In addition to these effects, ASA also produces important changes in the morphology of the cells and in their distribution in the cell cycle.

The work presented here expands our previous observations on the effect of NSAIDs, including ASA, on the kinetics of proliferation of colon cancer cells [8–10]. In our preceding work [10], ASA at concentrations up to 1.5 mM exerted an antiproliferative effect on HT-29 cultured colon cancer cells. However, because it failed to induce a subdiploid peak of cellular DNA content by FACS, DNA fragmentation on agarose gel electrophoresis, or characteristic morphologic features of apoptosis of cells stained with acridine orange, we concluded that ASA failed to induce apoptosis in these cells.

As our current data show, concentrations of ASA above 1.5 mM also demonstrated a time- and concentration-dependent antiproliferative effect on HT-29 cells. There is, however, evidence that a plateau may have been reached with 1 mM ASA at 72 hr. The effect on cell proliferation, which began 48 hr after ASA treatment, was preceded by its effect on the cell cycle. As early as 24 hr after ASA treatment, when no effect on cell proliferation could be perceived, the HT-29 cells accumulated in the G_0/G_1 phase. A similar pattern was observed when HT-29 cells were treated with other NSAIDs [9, 10, 27]. This temporal relationship between changes in the cell cycle distribution of the HT-29 cells and their number suggests an etiologic relationship between the two. We have also noted that these effects may be mediated by ASA-induced changes in the levels of key proteins that regulate the cell cycle [9, 10, 27].

Another principal observation made in this study was that, contrary to our previous findings with lower concentrations of this compound, ASA induces cell death by apoptosis in HT-29 colon adenocarcinoma cells. That apoptosis occurs in these cells in response to ASA treatment is clearly established by our new data generated from methods different from those we had used previously. Moreover, the EM photomicrographs establish this conclusion unambiguously. The methodology used in our previous study was relatively insensitive compared with the analyses used here and failed to detect ASA-induced apoptosis. Indeed, even the apoptosis induced by the higher concentrations of ASA used in the current study failed to produce a subdiploid peak on flow cytometric determination of

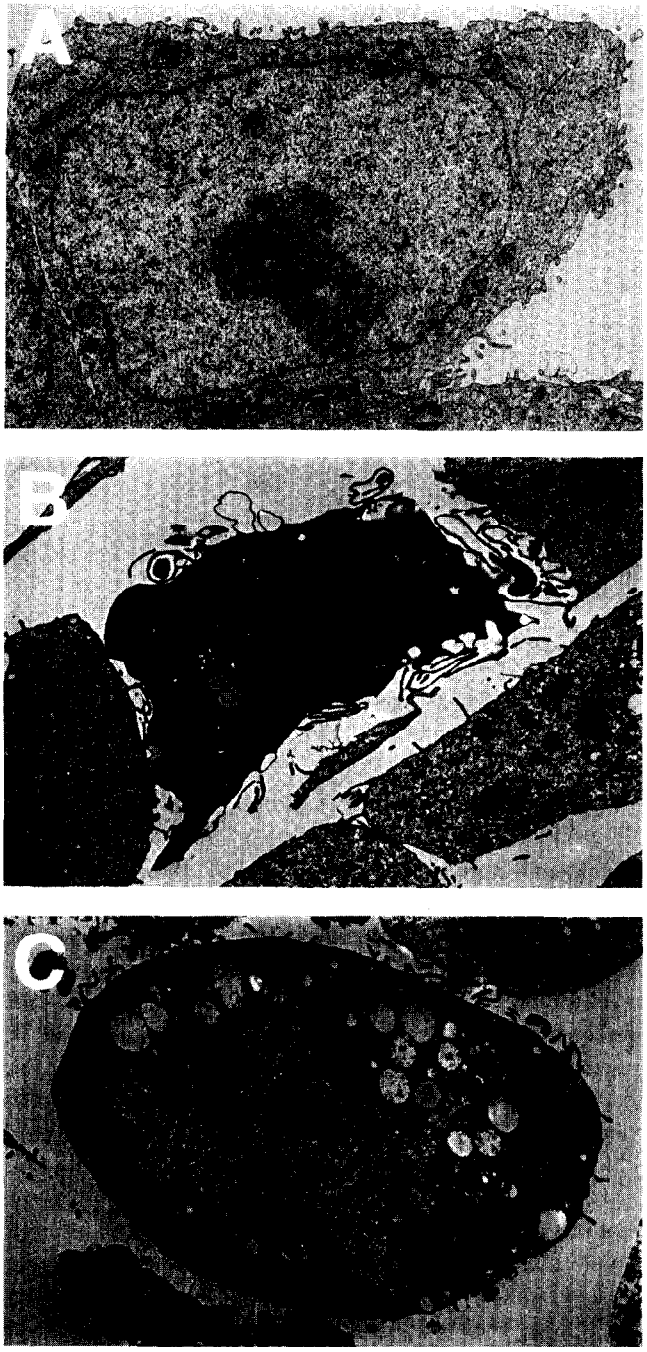

FIG. 9. Electron micrographs of aspirin-treated HT-29 cells. Control HT-29 cells (A), or those treated with 1 mM (B) or 3 mM (C) ASA for 72 hr, were examined by EM as in "Materials and Methods." ASA-treated cells show compacted nuclear chromatin with finely granular masses marginated against the nuclear envelope and condensed cytoplasm. The nuclear outline is convoluted. The organelles are preserved. These ultrastructural changes are characteristic of apoptosis. Magnification: 11,250×.

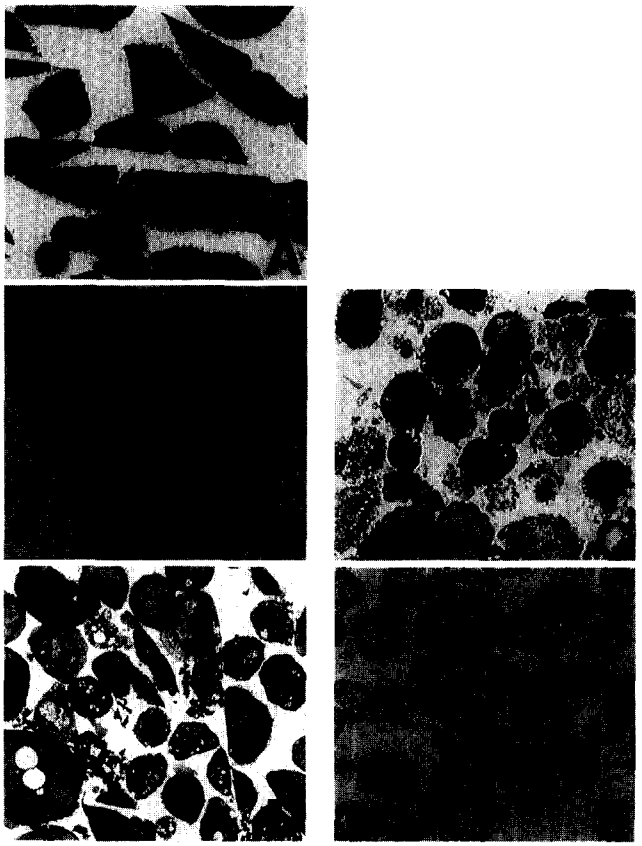

FIG. 10. Photomicrographs of semi-thin sections of aspirin-treated HT-29 cells. Semi-thin sections of control HT-29 cells (panel A), or those treated with 1 mM (panels B and C), or 3 mM (panels D and E) ASA for 72 hr, were examined as in "Materials and Methods." Panels A, B, and D show cells that remained attached to the culture dishes; panels C and E show the floating cell fractions from the corresponding plates. Attached HT-29 cells treated with 1 mM ASA (panel B) appeared identical to the controls (panel A), in contrast to the floating 1 mM ASA-treated HT-29 cells (panel C), some of which showed features of apoptosis. Cells with apoptotic features were present in both the attached (panel D) and floating (panel E) fractions from 3 mM ASA-treated HT-29 cells.

cellular DNA content or to generate an electrophoretic ladder.

The multiple indicators of apoptosis utilized in this study provide a more complete picture of the effect of ASA on the survival of HT-29 colon cancer cells: (a) apoptosis, assessed by DNA strand breaks, as detected with the TUNEL assay, occurs at low levels (~5%) by 24 hr of treatment, then increases to become a dominant event with longer exposure to this compound, involving over two-thirds (~67%) of the cells by 72 hr; (b) there appears to be a critical concentration threshold, around 1 mM, for apoptosis to become quantitatively significant; (c) the effect of ASA on apoptosis is independent of the cell cycle phase, implying that the target molecules for the effect of ASA on cell survival are present in all phases of the cell cycle; and (d) DNA strand breaks, as detected with the comet assay, are widespread in ASA-treated HT-29 cells, occurring twice as frequently as DNA nicks detected by TUNEL. This latter finding suggests either that relatively lengthy intermediate steps occur between DNA damage and the completion of apoptosis [28] or that repair mechanisms may be capable of reversing some of the ASA-induced DNA strand breaks.

An interesting aspect of the apoptosis induced by ASA is that it does not manifest all the conventional late stage morphological features. While it meets the stringent morphologic criteria for apoptosis by EM, apoptotic bodies were nevertheless characteristically absent in ASA-treated cells stained with DAPI. Apoptotic bodies were abundant in HT-29 cells treated with other NSAIDs such as sulindac sulfide, which was often used in parallel experiments (data not shown). Some apoptotic bodies were seen in semi-thin sections made from the floating fraction of HT-29 cells treated with ASA. The latter observation provides a mechanism for the disintegration of the apoptotic cell. But because apoptotic bodies were relatively rare, one suspects that either some additional pathways may be operative in this setting or they are not stable enough to be observed after the isolation process.

Several lines of evidence, outlined above, indicate that ASA induces a form of apoptosis in HT-29 cells that does not conform entirely with the classical features of this type of cell death. This may not be entirely surprising, as our appreciation of the subtleties of this phenomenon is still rather limited. The manifestations of apoptosis span a continuum where various combinations of features (including also features of necrosis) may be present in a particular instance [16, 29]. Therefore, based on our findings, ASA induces an "atypical" apoptosis in HT-29 colon cancer cells. However, the unusual features of ASA-induced apoptosis, besides their taxonomic value, may offer clues to the mechanisms that control the process of apoptosis or perhaps the cancer chemopreventive properties of this compound.

Comparison of the abilities of ASA and other NSAIDs to induce apoptosis is difficult. Detection of the apoptotic effect of ASA required different methodology than that required with other NSAIDs. It is not clear why HT-29 cells treated with various other NSAIDs, such as sulindac sulfide, indomethacin, or naproxen, manifested a subdiploid peak on flow cytometry but never after ASA treatment. One possible explanation is that ASA induces such extensive fragmentation of DNA that it generates very small DNA fragments or single nucleotides that cannot be observed with flow cytometry. On the other hand, the endonucleolytic activity of the other NSAIDs may be limited, compared with that of ASA, and thus lead to large DNA fragments that become apparent on both flow cytometry and agarose gel electrophoresis (the "DNA ladder"). Additional work clearly will be required to account for the differences between ASA- and non-ASA NSAID-induced apoptosis.

The relevance of these observations to colon carcinogenesis and colon cancer chemoprevention in humans needs to be determined. There are several unresolved issues related to, for example, the metabolic fate of ASA *in vivo* and in our culture system that preclude prudent extrapolation of our findings to its effect in humans. For instance, the various serine esterases that catalyze ASA hydrolysis can vary widely in terms of substrate specificity and K_m for ASA. Such esterases, known to occur in the intestinal wall [30], may or may not even be expressed by the cells we are studying. Another potential confounding factor is ASA's well-documented tight binding to and acetylation of albumin, a component of the fetal bovine serum added to the culture medium. Therefore, it is not valid to compare the ASA concentration used in our study to blood levels *in vivo* let alone the completely unknown colon tissue levels of ASA. (Therapeutic anti-inflammatory concentrations of salicylic acid, the immediate metabolite of ASA, range from 1–2 mM; at these levels, the concentration of ASA is about 0.1 mM.) It should be noted that we have used ASA *in vitro* at concentrations comparable to those studied by other investigators [31]. Nevertheless, our data clearly suggest that ASA has the *potential* of inducing apoptosis in colonocytes *in vivo*; such a possibility deserves examination by studies in animals and humans *in vivo*.

Determining if ASA induces apoptosis and inhibits proliferation of the epithelial cells in the human colon is potentially important. Tissue mass is critically affected by the rates of cell proliferation and cell death by apoptosis, and the colonic mucosa in both normal and pathological states is no exception [32–35]. On the other hand, the colon cancer preventive effects of ASA are well-documented [1]. Therefore, it is reasonable to speculate that the protective effect of ASA against colon cancer may be mediated, at least in part, by its effect on either one of these two mechanisms. Our work provides the theoretical basis for a detailed *in vivo* study to assess this possibility.

In summary, our work indicates that ASA profoundly affects the proliferation kinetics of cultured HT-29 human colon cancer cells by inhibiting their proliferation and inducing apoptosis, albeit somewhat differently from the apoptotic cell death induced by other NSAIDs. Since the antiproliferative and apoptotic effects of ASA may conceivably account for some of its chemopreventive effect in the colon, this possibility deserves further assessment.

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