Aspirin Effects on Endometrial Cancer Cell Growth

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Objective: **To find whether aspirin (acetylsalicylic acid, ASA) inhibits the growth of endometrial cancer cells in vitro in a way similar to that in colorectal cancer cells and to investigate the mechanisms by which aspirin might lead to growth inhibition.**

Methods: **Ishikawa human endometrial tumor cells were grown in the presence of ASA (1–5 mM) for 96 hours. Controls were treated with vehicle (absolute ethanol). Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide assay. Apoptosis was determined by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay. Analysis of cell-cycle distribution and bcl-2 expression was assessed by flow cytometry.**

Results: **Acetylsalicylic acid induced a dose-dependent inhibition of Ishikawa cells in vitro. The percentage of growth inhibition was 21–88% at concentrations of 1–5 mM. It also induced apoptosis and reduced bcl-2 expression in Ishikawa cells in a dose-dependent manner. Control cells and cells treated with the lowest concentration of ASA exhibited 2% apoptosis and more than 60% of the population expressed bcl-2. Apoptosis levels increased as levels of ASA increased from 2 to 5 mM (7–58%) with a concommitant decrease in bcl-2 expression from 46% at 2 mM to 2% at 5 mM. Acetylsalicylic acid concentrations of 3 mM or greater induced a shift from the resting phase (G0/G1) to S phase of the cell cycle.**

Conclusion: **Acetylsalicylic acid inhibited Ishikawa cell growth in vitro in a dose-dependent manner. Apoptosis is one of the mechanisms involved in the response, which can be mediated in part by downregulation of bcl-2. (Obstet Gynecol 2001;97:423–7. © 2001 by The American College of Obstetricians and Gynecologists.)**

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Experimental data suggest that aspirin (acetylsalicylic acid, ASA) and other members of the nonsteroidal anti-inflammatory drug family inhibit growth of cancer cells in vitro[1–4] and in vivo.[5,6] Clinical and epidemiologic data associated ASA and other nonsteroidal anti-inflammatory drugs with reduced occurrence and growth of certain tumors such as colon and breast cancers.[7,8]

In the United States, endometrial cancer is the most common malignacy of the female genital tract. The American Cancer Society estimated 37,400 new cases for 1999.[9] Although most endometrial cancers cancers are found at early stages, 6400 women were expected to die from it in 1999.[9] Most endometrial cancers (>75%) are of endometrioid histology.[10] Some investigators believe that type of endometrial cancer has several features in common with colon cancer and might share some pathogenetic mechanisms with it.[11] Based on that, we examined whether ASA inhibited growth of endometrial cancer cells in vitro similar to the way it did in colorectal cancer cells in vitro and in vivo. We also investigated potential mechanisms by which aspirin might mediate growth inhibitory effects.

Materials and Methods

The Ishikawa cell line was derived originally from a well-differentiated human endometrial carcinoma.[12] Those cells express estrogen and progesterone receptors and are well characterized in the literature.[13,14] Ishikawa cells were mantained in log phase growth in complete Leibowitz L-15 medium containing 200 mM L-glutamine, 20 μg/mL gentamicin, 20 mM HEPES (Gibco BRL, Gaithersburg, MD) and 10% heat-inactivated (56C, 30 minutes) charcoal-absorbed fetal bovine serum (Hyclone, Logan, UT). Ishikawa cells were grown in the presence of 1–5 mM ASA dissolved in ethanol (Sigma Chemical Co, St. Louis, MO) for 96 hours. Controls were incubated with vehicle (0.55% ethanol, Fisher Scientific, Fairlawn, NJ) added to me-

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dium to achieve the same concentration of ethanol as the treatment cultures. Cell growth was evaluated microscopically with a Leitz Ortholux inverted-phase microscope (Wild Leitz, Rockleigh, NJ). Confluent cultures were exposed to trypsin (0.05%) for 5 minutes at 37C after which the cells were washed and harvested for apoptosis and flow cytometry.

Acetylsalicylic acid was prepared fresh weekly as 1.0 M stock solutions in absolute ethanol. The stock solution was mixed with the culture medium to achieve concentrations of 1–5 mM ASA, with the final pH adjusted to 7.2. Absolute ethanol was added to the control cultures to achieve the maximum concentration of ethanol in the treatment cultures (0.55% ethanol). That concentration had negligible effects on growth, apoptosis, and expression of bcl-2 of the untreated Ishikawa cells.

The Promega cellTiter 96TM Cell Proliferation Assay (Promega, Madison, WI) was used to assess ASA-induced growth inhibition. Single cell suspensions of untreated Ishikawa cells were counted by hemocytometer and seeded in 96-well plates (Becton Dickinson, Lincoln Park, NJ) at 5×10^4 cells/well. Cells were grown at 37C with vehicle alone (controls) or 1–5 mM ASA. After 4 days of culture, 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide was added to the culture wells for 4 hours, after which cells were solubilized overnight at 37C; absorbance at 595 nm was recorded using an enzyme-linked immunosorbent assay plate reader (Titertek Multiscan Plus, Flow Laboratories, McLean, VA). All experiments were done in triplicate using eight wells for each treatment. Optical densities were compared between treatment groups and controls incubated with vehicle alone. The intra- and interassay coefficients of variation were less than 5% and 6%, respectively.

Apoptosis was indicated by terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling assay[15] using the Promega Apoptosis Detection System (Promega, Madison, WI) with fluorescein as described. Percentages of cells that showed fluorescein labeling of DNA fragments, indicative of apoptosis, were found with a Becton Dickinson FACScan Flow Cytometer (Mountain View, CA) using Lysis II software; green fluorescences of fluorescein-12-dUTP at 520 nm and red fluorescence of propidium iodide at 620 nm were measured.

Bcl-2 protein was detected by flow cytometry as described.[16] Ishikawa cells were fixed in 1.0% paraformaldehyde (Sigma Chemical Co.) and fixed cells were suspended in a solution of 0.3% saponin in phosphate buffered solution (Sigma Chemical Co.) containing bcl-2 monoclonal antibody (clone 124, Dako, Santa Barbara, CA). After incubation at 0C for 30 minutes, cells were washed by centrifugation and incubated an additional 30 minutes at 0C with phycoerythrin-labeled rat anti-murine immunoglobulin (Becton Dickinson, Lincoln Park, NJ). Percentages of cells that exhibited positive staining were found with a FACScan flow cytometer with Lysis II software.

DNA cell-cycle analysis of cultured cells was done according to a modified Krishan procedure.[17] Cells were grown in culture medium treated with vehicle and 1–5 mM ASA for 96 hours. Analyses of S-phase fraction were expressed using FACScan Cellfit software for doublet discrimination and expressed as the DNA index and percentage of the population in G0/G1, S, and G2 + M phases.

Statistical analysis was done with SigmaStat software (Jandel Scientific, San Rafael, CA). Cell proliferation data were expressed as percentage inhibition of control culture proliferation (mean optical density of wells treated with vehicle alone), which was taken as 100%. Standard errors were within 10% of the mean of replicate wells. All relevant comparisons were made within one bioassay or immunoassay. Sample size was calculated to detect a difference in the means of 20% with an alpha of .05 and a power of 0.845. Data were analyzed by one-way and Kruskal–Wallis analyses of variance. For data not normally distributed, all pairwise multiple comparison procedures (Student–Neuman–Keuls and Dunnett methods) were used to isolate the groups that differed from the others; $P < .05$ was considered significant. Dose-dependent relations were determined by multiple linear regression.

Results

The effects of ASA on growth of Ishikawa endometrial cancer cells after 96 hours of treatment are shown in Figure 1. Acetylsalicylic acid induced a dose-dependent inhibition of Ishikawa cell growth in the range of 1–5 mM. Greater than 50% mean inhibition was achieved at ASA concentrations of 3 mM and almost complete suppression was seen at 5-mM concentrations. The standard error of the mean for all concentrations was well below 10%.

Apoptosis was negligible in cells treated with vehicle alone and in cells exposed to 1- or 2-mM concentrations of ASA. The concentration of ASA present in the medium was increased beyond 2 mM, so apoptosis inducement was dose dependent. In cultures treated with 3 mM ASA, more than 25% of the cells stained positive for DNA fragmentation indicative of apoptosis (Table 1). After exposure to 5 mM ASA, nearly 60% of cells showed apoptosis.

Preliminary studies found that untreated Ishikawa cells exhibit high expression of the apoptosis inhibiting protein bcl-2. Apoptosis was induced by ASA treatment, so we investigated whether bcl-2 expression was

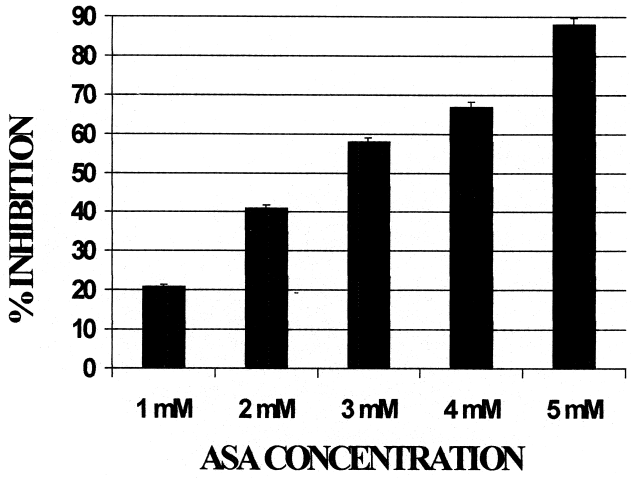

Figure 1. Dose-dependent inhibition of Ishikawa cells by ASA present at different concentrations (1–5 mM) for 96 h, compared with growth of controls treated with vehicle alone. Values represent mean ± SE. $P < .05$ for all groups compared with controls ($R = .89$). ASA = acetylsalicylic acid.

altered in the treated cultures. Expression of bcl-2 protein was assessed simultaneously with the measurement of apoptosis in Ishikawa cells exposed to increasing concentrations of ASA (Table 1). In control cultures, bcl-2 expression was nearly 70%, with negligible apoptosis noted. Similar results were observed in cultures exposed to 1 mM ASA. At 2 mM ASA, the expression of bcl-2 remained close to 50%, whereas apoptosis was less than 10%. As the concentration of ASA increased beyond 2 mM, the percentage of cells that expressed bcl-2 was statistically decreased to levels below 20%, whereas apoptosis was increased to nearly 30% or greater in those cultures. At concentrations of 5 mM, the expression of bcl-2 was less than 5% and nearly 40% of the cell populations in those cultures were undergoing apoptosis.

Treatment with ASA concentrations of 3 mM or greater induced a shift of cells from the resting phase (G0/G1) to the S phase of the cell cycle (Table 2). That effect was in conjunction with a significant decrease in the expression of bcl-2 protein and coincident increase in apoptosis (Table 1).

Table 1. Mean Apoptosis and bcl-2 Expression After Incubation With Aspirin or Vehicle for 96 Hours

	Control	1 mM ASA	2 mM ASA	3 mM ASA	4 mM ASA	5 mM ASA
Mean (SE) %apoptosis	2 (0)	2 (1)	5 (2)	19* (8)	25* (5)	36* (7)
Mean (SE) %bcl-2 expression	67 (7)	65 (2)	49 (3)	22* (5)	10* (4)	2* (0)

ASA = acetylsalicylic acid; SE = standard error of the mean.
* $P < .05$ compared with control.

Table 2. Cell-Cycle Phase Distribution of Ishikawa Cells Treated With Aspirin for 96 Hours

Treatment	%G0/G1	%S	%G2 + M
Control	63 ± 2	26 ± 1	11 ± 1
1 mM ASA	67 ± 1	25 ± 1	8 ± 1
2 mM ASA	68 ± 1	25 ± 1	7 ± 1
3 mM ASA	61 ± 8	35 ± 14	4 ± 1
4 mM ASA	44 ± 13	50 ± 14	6 ± 1
5 mM ASA	48 ± 4	47 ± 3	5 ± 3

Values represent mean ± standard error for triplicate studies.
ASA = acetylsalicylic acid; G0/G1 = resting phase; S = DNA synthesis phase; G2 + M = mitosis.

Discussion

Colorectal and endometrial cancers have many features in common. Both have identifiable histopathologic precursor lesions. Histopathologic progression from adenoma to carcinoma in colon cancer also is seen in the atypical hyperplasia–endometrial carcinoma sequence. Colorectal and endometrioid adenocarcinoma of the uterus also have similar histologic appearances. Certain alterations in oncogenes and tumor supressor genes, such as p53 and K-ras, in colorectal cancer also are seen in endometrial cancer.[18,19] Microsatellite instability in colorectal cancer[20] also has been found in endometrial cancer.[11] In women of families afflicted with hereditary nonpolyposis colorectal cancer of the Lynch syndrome variant, endometrial carcinoma is the most common noncolorectal malignancy reported.[21] Based on those similarities, we postulated that nonsteroidal anti-inflammatory drugs also inhibit growth of an endometrial carcinoma cell line in way a similar to that described for colorectal cancer. Our study shows a dose-dependent inhibition of Ishikawa cell growth by ASA in the range of 1–5 mM. Those results compare favorably to data of Elder et al[4] who examined exposure of colon adenocarcinoma cell lines to the same concentrations of sodium salicylate.

Several mechanisms of growth inhibition by nonsteroidal anti-inflammatory drugs on colon cancer cell lines have been suggested, including reduction of the rate of cellular proliferation and induction of programmed cell death, or apoptosis.[3,4] In this study, the growth inhibitory effect of ASA seems to be mediated in part by induction of apoptosis. The gene product of bcl-2, originally described in non-Hodgkin lymphomas,[22] enhances cell survival by inhibiting apoptosis.[23] Several studies found bcl-2 in normal endometrium.[24,25] Bcl-2 expression in glandular cells peaks at the end of the follicular phase and disappears in the late secretory phase. The effect of bcl-2 in this setting might be prevention of apoptosis during the proliferative phase. In malignant endometrium, bcl-2 expression was highly correlated to hormone receptors and tumor differentia-

tion and endometrioid versus papillary subtypes.[26,27] However, some authors reported decreased bcl-2 expression in endometrial adenocarcinoma.[28] To examine whether apoptosis was one possible mechanism involved in the antiproliferative effect induced by ASA, we found the rate of apoptosis and bcl-2 expression in Ishikawa cells exposed to ASA in vitro. We found that as the concentration of ASA is increased there is a rise in the rate of apoptosis and a reciprocal decrease in the rate of bcl-2 expression.

DNA analysis found that elevated concentrations of ASA induced a shift from resting to proliferative phase of the cell cycle, coincident with the induction of apoptosis. That effect was observed with a variety of chemotherapeutic drugs[29–31] and other agents including caffeine[32] and tumor promoters such as okadaic acid[33] and staurosporine.[34] Although nonsteroidal anti-inflammatory drugs including salicylate, piroxicam, naproxen, and sulindac induced similar responses in colon cancer cells in a concentration-dependent manner, those agents also reduced cell growth by increasing the fraction of cells in G0/G1 phases of the cycle.[3,4,35] Aside from cell-cycle effects, the most commonly reported observation after nonsteroidal anti-inflammatory drug exposure is induction of apoptosis. In vivo, sulindac inhibited mammary[36] and colon[37] carcinogenesis in rats by the induction of apoptosis. In vitro, sulindac-induced apoptosis was associated with cell-cycle quiescence as a function of significantly decreased levels of several cyclin-dependent kinases[1,34] and reduced levels of the mitotic cyclin proteins A and B and cyclin inhibitor protein $p21^{wafl/cipl}$.[3] We found that increased levels of apoptosis induced by ASA in vitro correlated with reduced expression of bcl-2 protein, and additional studies are examining the effect of cell-cycle proteins in that inhibitory response.

The effects of ASA on endometrial cancer cell growth are not surprising. Numerous cell lines and malignancies have been the subject of experimentation with ASA and other nonsteroidal anti-inflammatory drugs. Most studies confirmed the potential of ASA and related compounds as chemopreventive agents for malignancies such as colon carcinoma. To fully exploit the potential of ASA in chemoprevention, one must first understand how ASA inhibits the growth of neoplastic cells. Unfortunately, the mechanism is not entirely clear. Initially, it was believed that inhibition of prostaglandin synthesis was involved. The demonstration that upregulation of cyclo-oxygenase-2 in human colorectal carcinomas and adenomas appears to be involved in development of colon cancer lent credence to that theory.[38] However, that simple explanation was challenged by findings that sulindac sulfone, a metabolite of sulindac that lacks prostaglandin inhibitory abilities, is capable of inhibiting the growth of colon cancer cells[2] by apoptosis. This was further supported by Hanif et al[39] who found that effects of nonsteroidal anti-inflammatory drugs on two colon cancer cell lines were independent of prostaglandin synthesis. How prostaglandin inhibition and apoptosis interact is not entirely clear. Some authors have suggested that cyclo-oxygenase-2 overexpression might enable cells to escape apoptosis and lead to malignant transformation.[40] That was shown by Sheng et al[41] who showed that forced expression of cyclo-oxygenase-2 leads to inhibition of apoptosis in intestinal epithelial cells. Moreover, those investigators found that prostaglandin E_2 inhibits programmed cell death caused by a selective cyclo-oxygenase-2 inhibitor and induces bcl-2 expression. More recently, Tsuji et al[42] found a separate effect of aspirin on colon cancer cells in vitro. In a colon cancer in vitro model, cyclo-oxygenase regulated colon carcinoma–induced angiogenesis by two mechanisms: cyclo-oxygenase-2 can modulate production of angiogenic factors in those cells; and cyclo-oxygenase-1 regulates angiogenesis in endothelial cells. Both enzymes and their effects were inhibited by aspirin. It might be that, in vivo, inhibition of prostaglandin synthesis and apoptosis are both necessary to reverse the malignant phenotype.

Endometrial carcinoma is an ideal cancer for chemoprevention because it is common and has a known, symptomatic, and demonstrable preinvasive phase. There are many known risk factors that predispose certain populations to this disease including unopposed estrogen, obesity, tamoxifen therapy for breast cancer, a history of other malignancies such as breast, colon, and ovarian cancer, and atypical endometrial hyperplasia. If the effects of ASA on endometrial cancer can be shown in animal models and humans, populations at risk could be targeted in future chemoprevention trials in hopes of decreasing the incidence of this disease.

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