Effects of Nonsteroidal Anti-Inflammatory Agents (NSAIDs) on Ovarian Carcinoma Cell Lines: Preclinical Evaluation of NSAIDs as Chemopreventive Agents[1]

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ABSTRACT

Purpose: **Nonsteroidal anti-inflammatory agents may inhibit carcinogenesis in specific tissues including the colon, breast, and pancreas, and, hence, may prove to be effective chemopreventive agents. The purpose of this study was to investigate the cellular effects of acetylsalicylic acid (ASA), acetaminophen, and a COX-2 inhibitor (NS-398) on the growth of cell lines of human ovarian cancer *in vitro*. *Experimental Design:* SK-OV-3, Caov-3, and NIH:OVCAR-3 ovarian carcinoma cell lines were treated with ASA (10^{-6} M—10^{-2} M), acetaminophen (10^{-6} M—10^{-2} M), and a COX-2 inhibitor (10^{-6} M–10^{-4} M) for 96 h. The number of viable cells was determined using a tetrazolium conversion assay. Immunohistochemical assessment was performed for alterations in expression of Ki-67, erbB-2, COX enzyme, and apoptosis in primary ovarian cancer cells using terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling assay. *Results:* A decrease in cell number compared with controls was observed for all of the cell lines treated with ASA, acetaminophen, and COX-2 inhibitor by cell count and tetrazolium conversion assay. A significant decrease in Ki-67 compared with controls in the OVCAR-3 ($P = 0.005$) and SK-OV-3 ($P = 0.007$) cell lines after treatment with the COX-2 inhibitor was observed. We observed a decrease in mitotic activity compared with controls in each cell line after treatment with the COX-2 inhibitor. Apoptosis was observed in primary ovarian cancer cell culture treated with COX-2 inhibitor. *Conclusion:* Our results suggest additional study for the use of nonsteroidal anti-inflammatory agents, specifically COX-2 inhibitors, as a strategy of chemoprevention for ovarian cancer.**

INTRODUCTION

Ovarian carcinoma represents an insidious disease that typically has progressed to an advanced stage at the time of diagnosis. As such, advances in therapeutic interventions have had little impact on the long-term reductions in deaths attributable to ovarian cancer. Moreover, secondary to the low prevalence of disease, screening strategies have suffered from prohibitively high rates of false positive tests and, hence, have not proved to be useful clinically (1, 2). Therefore, to attain a goal of reductions of death because of disease, prevention of ovarian carcinoma represents the most rational strategy (1).

Chemopreventive strategies have been investigated in a variety of other solid tumors; chemoprevention trials in breast, colon, head and neck, and liver cancers suggest the potential utility of such an approach using a variety of agents (3–7). Additionally, utilization of nonsteroidal anti-inflammatory compounds (NSAIDs)[3] in familial polyposis and retinoids in oral dysplasia suggest the ability of chemopreventive agents to interrupt the sequence of carcinogenic events leading to an invasive malignancy (3, 8).

Epidemiological and experimental data have supported the use of NSAIDs as potential chemopreventive agents in a variety of types of cancer. Although some epidemiological data indicate that NSAIDS may be effective in the prevention of ovarian cancers (9, 10), little data are available regarding the effects of NSAIDS on cell lines of ovarian carcinoma. Therefore, we evaluated three common types of NSAIDs in regard to their ability to inhibit the growth of ovarian tumor cells.

MATERIALS AND METHODS

Cell Lines and Conditions. NIH:OVCAR-3, SK-OV-3, and Caov-3 cell lines (American Type Culture Collection, Manassas, VA) were maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) containing 10% fetal bovine serum (Life Technologies, Inc.), 2 mM L-glutamine (Mediatech, Herndon, VA), antibiotic-antimycotic solution (1X; Mediatech), and MEM vitamin solution (1X; Mediatech). Each cell line was

Received 5/9/01; revised 8/15/01; accepted 10/3/01.
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[1] Supported in part by a grant from the Specialized Program of Research Excellence (SPORE) in Ovarian Cancer NCI CA83591-02 and the University of Alabama at Birmingham Estes Fund for Gynecologic Cancer Research.
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[3] The abbreviations used are: NSAID, nonsteroidal anti-inflammatory drug; COX, cyclooxygenase; ASA, acetylsalicylic acid; TUNEL, terminal deoxynucleotidyl transferase (Tdt)-mediated nick end labeling; CI, confidence interval; FSH, follicle-stimulating hormone.

maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Tetrazolium Conversion Assay. After 24 h in culture, each cell line was treated with ASA (Sigma Chemical Co., St. Louis, MO) and acetaminophen (Sigma Chemical Co.) at concentrations from 10^{-6} to 10^{-2} M in DMSO for 48, 96, and 120 h. Each cell line also was treated with a COX-2 inhibitor (Cayman Chemical, Ann Arbor, MI) at concentrations from 10^{-6} to 10^{-4} M for 48, 96, and 120 h. Cells were plated in quadruplets at each concentration. The same experiment was repeated with each cell line treated with ASA, acetaminophen, and the COX-2 inhibitor at the same concentrations for 96 h in 1% absolute ethanol. The maximum concentration tested for the COX-2 inhibitor was 10^{-4} M because of limitations in its solubility in DMSO and in 1% absolute ethanol. Cell number was determined using a tetrazolium conversion assay (Promega, Madison, WI).

Preparation of Cell Lines for Immunohistochemistry. Each cell line was plated on coverslips as described previously (11) such that in 5 days the confluency of each cell line would range between 55 and 65%. After 24 h, ASA, acetaminophen, and the COX-2 inhibitor were added to each cell line at 10^{-4} M. The control group for each cell line was treated with 1% ethanol. Each cell line was also plated without the vehicle or any agent. After 96 h, the cells were rinsed in PBS two times and fixed in neutral-buffered formalin.

Immunohistochemistry. Standard immunostaining procedures as described previously (12–14) were used to determine the expression of various biomarkers. The cells on the coverslips were permeabilized using acetone (Fisher Scientific, Norcross, GA) followed by graded (absolute to 70%) ethanols to remove the acetone and were returned to a Tris buffered bath (0.05 M Tris base, 0.15 M NaCl, 0.002% Triton X-100, pH 7.6). Endogenous peroxidases were quenched using an aqueous solution of 3% H_2O_2 for 5 min. Goat serum (3%) was added for 1 h at room temperature to block nonspecific immunostaining. The coverslips were incubated with the appropriate primary antibody for 1 h at room temperature. The primary antibodies used were Ki-67 (clone MIB-1; Biogenex, San Ramon, CA), erbB-2 (clone 3B5; Oncogene Sciences, Cambridge, MA), COX-1 (Cayman Chemical), and COX-2 (Cayman Chemical) at concentrations of 3 μg/ml and 0.5 μg/ml, and dilutions of 1:200 and 1:100, respectively. Primary antibodies were detected using a multispecies detection system from Signet Laboratories, Inc. (Dedham, MA). After the removal of the primary antibodies by washing, the cells were incubated in an antimouse/antirabbit antibody for 10 min and washed. Then a peroxidase-labeled streptavidin was added for 5 min followed by washing. A 3,3′-diaminobenzidine super sensitive substrate kit (Biogenex) was used to visualize the antibody-antigen complex. The coverslips were then counterstained using hematoxylin, dehydrated using graded ethanols, and soaked with xylene before attaching the coverslip to a microscope slide using Permount.

Immunohistochemistry Evaluations. For Ki-67, the percentage of staining nuclei was estimated by counting positive and negative nuclei on 15 random fields for each coverslip for all of the cell lines undergoing each treatment. Also, the total cell count (cells with positive and negative nuclei) was determined. For erbB-2, COX-1, and COX-2, an immunohistochemistry score was assigned such that tumor cells were classified in regard to percentage of cells stained at each intensity from 0 (no staining) to 4 (highest intensity staining). A cytoplasmic and membrane immunostaining score was determined for erbB-2. A cytoplasmic and nuclear immunostaining score was evaluated for COX-1, and a cytoplasmic, nuclear, and membrane immunostaining score was evaluated for COX-2. Presence of mitotic activity was analyzed per 15 random fields on each coverslip for all of the cell lines with each treatment. In addition, as above, the total number of cells in 15 random fields were assessed while determining mitotic activity.

Organ Cultures. Five primary ovarian tumors through the University of Alabama at Birmingham Ovarian Specialized Program of Research Excellence tissue resource were obtained from patients undergoing surgery for ovarian cancer. Human ovarian carcinoma tissue from each tumor was cut in tiny pieces (<1 mm) and was exposed to medium alone, 1% absolute ethanol (vehicle), and the COX-2 inhibitor (10−4 M) for 4 h, 8 h, and 40 h. After exposure to each treatment, ovarian carcinoma tissues were fixed in neutral-buffered formalin and processed to paraffin blocks.

TUNEL Assay. Organ cultures were assessed for apoptosis using a TUNEL assay. Paraffin sections (5 μm) of tissue were mounted on Superfrost/Plus slides and attached by heating at 58°C for 1 h. Tissue sections were deparaffinized in three changes of xylene and rehydrated with one change of 100% ethanol, 95% ethanol, and 70% ethanol, each in 5-min increments. Then, the sections were placed in Tris-buffered saline [(0.05 M Tris base, 0.15 M NaCl, and 0.0002% Triton X-100 (pH 7.6)].

The protocol for staining apoptotic nuclei was performed using an ApopTag Peroxidase kit (Intergen, Purchase, NY). Proteinase K (20 μg/ml in distilled deionized H_2O) was added to the tissue specimens and incubated at room temperature for 15 min. Endogenous peroxidases were quenched with an aqueous solution of 3% hydrogen peroxide for 5 min. Sections were treated with an equilibration buffer (Intergen) for 30 min and then incubated with the TdT enzyme (diluted in a labeling reaction mix) for 1 h at 37°C using parafilm covers. At this point, the TdT enzyme binds 3′-OH ends of DNA fragments and catalyzes the addition of digoxigenin-labeled and unlabeled deoxynucleotides. Negative controls were incubated with deionized H_2O (diluted in labeling reaction mix) instead of the TdT enzyme. Positive controls included a multitissue block with tumor and normal tissue.

A stop buffer (Intergen) was added for 10 min at room temperature to terminate the labeling reaction. An antidigoxigenin conjugate was added to each slide for 30 min. The chromagen 3,3′-diaminobenzidine was used to visualize the labeled 3′-OH end of DNA fragments. The slides were then rinsed in deionized water and lightly counterstained with hematoxylin, dehydrated, and coverslipped with Permount.

Statistical Analysis. The percentage of cells staining positive for Ki-67 in each treatment group was compared with the control group for each cell line using a comparison of binomial proportions. The significance tests were 2-sided with an α level equal to 0.05. Similarly, the percentage of mitotic activity in each treatment group was compared with the control group using a comparison of binomial proportions.

The mean absorbance values from the tetrazolium conversion assay were determined for the control group and the drug

treatment groups for each cell line. A general linear model approach to ANOVA for unbalanced, normally distributed data was used to compare the mean absorbance values of the control and drug treatment groups while controlling for the effect of concentration. This procedure was performed for each cell line.

The total number of cells on 30 random fields as described previously was used to determine for each treatment group and control group for each cell line. Comparisons between the mean total number of cells (not normally distributed) in the control group *versus* the treatment groups for each cell line were conducted using the Wilcoxon two-sample test. Analyses were performed using SAS statistical software programs (15).

Western Blot Analysis Using Chemiluminescence. Cell lysates of the OVCAR-3, SK-OV-3, and Caov-3 cell lines were used in a Western blot analysis using chemiluminescence. COX-1 and COX-2 was separated by SDS-PAGE as described by Laemmli (16). The Bio-Rad (Hercules, CA) mini-gel system was used for this procedure as reported previously (17). Briefly, protein concentrations of the cell lysates were determined using a Pierce bicinchoninic acid protein assay kit (Pierce, Rockford, IL) in order that equal concentrations of each protein were loaded onto each lane of the gel. The proteins were electrotransferred to nitrocellulose membranes (0.45 μm) as reported by Towbin *et al.* (18). The membranes were immersed in 5% milk block buffer [(pH 7.5) 5% powdered milk, 0.02% NP40, 0.15 M NaCl, and 0.02 M Tris] overnight at 4°C. After washing the membranes in Tris-buffered saline buffer [(pH 7.6) 20 mM Trizma Base, 137 mM NaCl, 1 M HCl, and dH_2O] with 0.1% Tween, the membranes were incubated with monoclonal antibodies to COX-1 (Cayman Chemical) and COX-2 (Cayman Chemical) overnight at 5 μg/ml and 1 μg/ml, respectively, on a shaker at 4°C. The blots were then rinsed and incubated with secondary antimouse horseradish peroxidase at 1:3000 (Bio-Rad) for 1 h at room temperature. After rinsing the blot, the enhanced chemiluminescence Western blot system (Amersham Pharmacia Biotech, Piscataway, NJ) was used for immunodetection, and the blot was exposed to Hyperfilm enhanced chemiluminescence (Amersham) for visualization of the antigen-antibody complex.

RESULTS

Fig. 1 shows the number of viable cells determined using a tetrazolium conversion assay on OVCAR-3, SK-OV-3, and Caov-3 cell lines after treatment with ASA, acetaminophen, and the COX-2 inhibitor in 1% absolute ethanol for 96 h. A decrease in cell number compared with controls was observed at concentrations of 10^{-3} to 10^{-2} M for all of the cell lines treated with ASA and acetaminophen. A similar decrease in cell number compared with controls was observed from concentrations of 10^{-5} M to 10^{-4} M in each cell line treated with the COX-2 inhibitor. The same patterns of growth inhibition shown in Fig. 1 were also observed in each cell line after treatment with ASA, acetaminophen, and the COX-2 inhibitor at the same concentrations using DMSO as a vehicle at 48, 96, and 120 h.[4] However, these results using DMSO were complicated as were

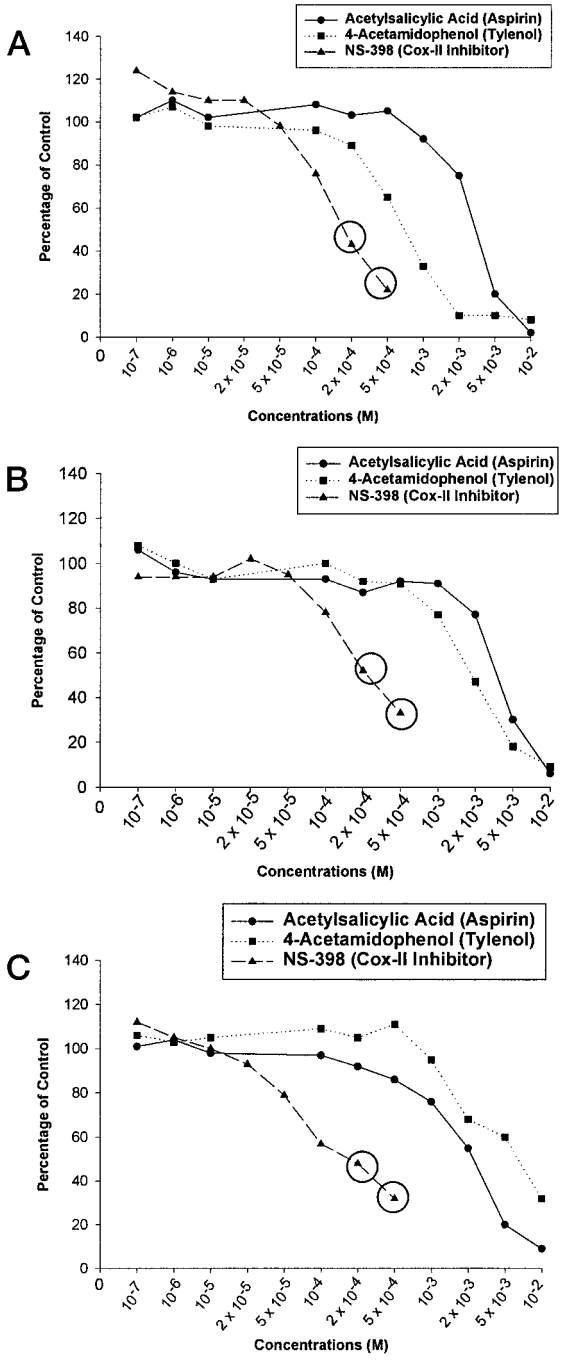

Fig. 1 Cell growth curves expressed as percentage of control for Caov-3, OVCAR-3, and SKOV-3 cell lines after treatment with varying concentrations of ASA (*aspirin*), 4-acetamidophenol (*Tylenol*), and NS-398 (*COX-2 inhibitor*) for 96 h. *A*, total number of Caov-3 cells expressed as percentage of control after treatment with varying concentrations of ASA (*aspirin*), 4-acetamidophenol (*Tylenol*), and NS-398 (*COX-2 inhibitor*) for 96 h. *B*, total number of OVCAR-3 cells expressed as percentage of control after treatment with varying concentrations of ASA (*aspirin*), 4-acetamidophenol (*Tylenol*), and NS-398 (*COX-2 inhibitor*) for 96 h. *C*, total number of SKOV-3 cells expressed as percentage of control after treatment with varying concentrations of ASA (*aspirin*), 4-acetamidophenol (*Tylenol*), and NS-398 (*COX-2 inhibitor*) for 96 h. The data points that are circled are concentrations of COX-2 that exceeded solubility limits. Each growth curve presented was repeated at least two times.

[4] Unpublished observation.

Table 1 Mean absorbance values[a] obtained from a tetrazolium conversion assay measuring total number of viable cells

	Control group[b]	ASA	Acetaminophen	COX-2 inhibitor
Caov-3 cell line	0.611 ± 0.014[c]	0.576 ± 0.042	0.389 ± 0.041[d]	0.405 ± 0.029[d]
OVCAR-3 cell line	0.253 ± 0.005	0.210 ± 0.015[d]	0.235 ± 0.011	0.158 ± 0.010[d]
SKOV-3 cell line	0.682 ± 0.009	0.552 ± 0.036[d]	0.510 ± 0.038[d]	0.489 ± 0.026[d]

[a] The higher the mean absorbance value, the higher the total number of viable cells.
[b] The control group used contained the vehicle, 1% absolute ethanol.
[c] Mean absorbance value ± SE mean.
[d] Statistically significant ($P < 0.05$; General Linear Model procedure) compared with control values.

Table 2 A comparison of least square mean absorbance values obtained from a tetrazolium conversion assay measuring total number of viable cells across drug treatment groups in ovarian carcinoma cell lines

	Caov-3 cell line[a]	OVCAR-3 cell line[b]	SKOV-3 cell line[c]
ASA *vs.* acetaminophen	<0.0001[d]	0.0134[d]	0.1087[d]
ASA *vs.* COX-2 inhibitor	<0.0001[d]	<0.0001[d]	<0.0001[d]
Acetaminophen *vs.* COX-2 inhibitor	<0.0001[d]	<0.0001[d]	<0.0001[d]

[a] Least square mean absorbance values for ASA, acetaminophen, and COX-2 inhibitor groups were 0.6387, 0.4522, and 0.2484, respectively.
[b] Least square mean absorbance values for ASA, acetaminophen, and COX-2 inhibitor groups were 0.2295, 0.2542, and 0.1092, respectively.
[c] Least square mean absorbance values for ASA, acetaminophen, and COX-2 inhibitor groups were 0.6071, 0.5649, and 0.3508, respectively.
[d] *P* were obtained using a General Linear Model procedure.

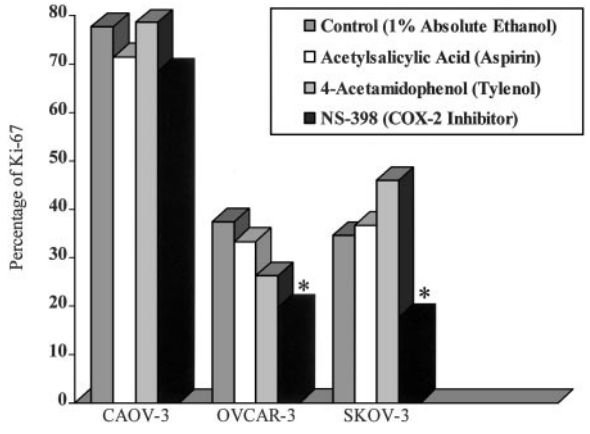

Fig. 2 Percentage of nuclei expressing Ki-67 through immunohistochemical methods in Caov-3, OVCAR-3, and SKOV-3 cells treated with the same concentration (10^{-4} M) of ASA (*aspirin*), 4-acetamidophenol (*Tylenol*), and NS-398 (*COX-2 inhibitor*) for 96 h. A comparison of binomial proportions was conducted with significance tests that were 2-sided with an α level equal to 0.05. The percentage of Ki-67 in each cell line receiving each treatment (ASA, 4-acetamidophenol, and NS-398) was compared with the control group which contained the vehicle, 1% absolute ethanol. ∘ = A; $P < 0.01$.

the growth curves, because DMSO alone stimulated cell growth compared with medium without DMSO (19).

A comparison of the mean absorbance values between the control group and the treatment groups in each cell line was evaluated using a general linear model procedure. Statistically significant ($P < 0.05$) differences between the mean absorbance values of the control group and each treatment group were observed in the SKOV-3 cell line. For the Caov-3 cell line, statistically significant differences ($P < 0.05$) between the mean absorbance values of the control group and the acetaminophen and COX-2 inhibitor groups were observed. For the OVCAR-3 cell line, statistically significant differences ($P < 0.05$) between the mean absorbance values of the control group and the ASA and COX-2 inhibitor groups were observed. Table 1 includes the mean absorbance values for each control and drug treatment group for each cell line. Using the general linear procedure, a comparison of the least square mean absorbance values was compared across all of the drug treatment groups. Controlling for concentration, drug treatment was a significant predictor of absorbance. Overall, statistically significant ($P < 0.01$) differences were observed between drug treatment groups in each cell line. The only comparison that was not statistically significant was the least square mean absorbance values between the acetaminophen and ASA groups in the SKOV-3 cell line. Table 2 indicates the least square mean absorbance values for each group in each cell line.

After exposing each cell line for 96 h to ASA, acetaminophen and the COX-2 inhibitor at concentrations of 10^{-4} M, coverslips were immunostained for Ki-67, erbB-2, COX-1, and COX-2. The total number of cells staining positive and negative for Ki-67 per slide was determined. A significant decrease in Ki-67 compared with controls in the OVCAR-3 ($P = 0.005$) and SK-OV-3 ($P = 0.007$) cell lines after treatment with the COX-2 inhibitor was observed. Although not significant, a decrease in Ki-67 compared with controls was observed for Caov-3 cells ($P = 0.139$) treated with the COX-2 inhibitor. Fig. 2 includes the percentage of cells positive for Ki-67 with each treatment. The changes in Ki-67 expression in each cell line after treatment with the COX-2 inhibitor may be correlated with changes in mitotic activity. Although not significant, we observed a decrease in mitotic activity compared with controls in each cell line after treatment with the COX-2 inhibitor. The percentage of cells undergoing mitotic activity in the control group and the percentage of cells undergoing mitotic activity after treatment with the COX-2 inhibitor for Caov-3, OVCAR-3, and SKOV-3 cells were 3.37% and 2.65% ($P = 0.766$); 1.43% and 0.936% ($P = 0.747$); and 2.15% and 1.43% ($P = 0.701$), respectively. Similarly, the total number of cells attached to coverslips on 30 random fields was determined and

Table 3 A comparison of the mean total number of viable cells in the control group *versus* drug treatment groups in ovarian carcinoma cell lines[a]

	Control group[b]	ASA	Acetaminophen	COX-2 inhibitor
Caov-3 cell line	179 ± 31[d]	152 ± 19	132 ± 28[c]	88 ± 10[c]
OVCAR-3 cell line	69 ± 5	67 ± 7	79 ± 7	57 ± 4[c]
SKOV-3 cell line	134 ± 6	124 ± 11	118 ± 6	88 ± 6[c]

[a] The total number of cells on 30 random fields was determined on cells attached to coverslips.
[b] The control group used contained the vehicle, 1% absolute ethanol.
[c] Mean ± SE mean.
[d] Statistically significant ($P < 0.05$; Wilcoxon two-sample test) compared with control values.

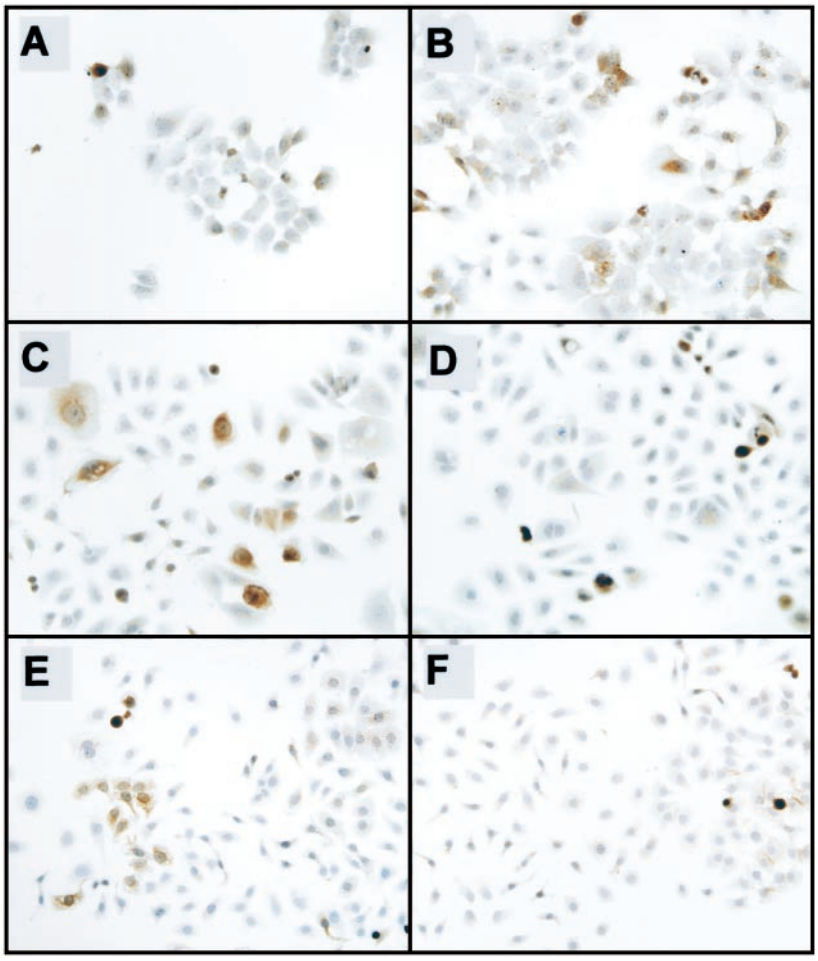

Fig. 3 COX-1 and COX-2 protein expression in untreated Caov-3, OVCAR-3, and SKOV-3 cells through immunohistochemical methods. *A*, COX-1 expression in untreated Caov-3 cells. *B*, COX-2 expression in untreated Caov-3 cells. *C*, COX-1 expression in untreated OVCAR-3 cells. *D*, COX-2 expression in untreated OVCAR-3 cells. *E*, COX-1 expression in untreated SKOV-3 cells. *F*, COX-2 expression in untreated SKOV-3 cells.

compared with controls (Table 3). For each cell line, a significant decrease ($P < 0.05$) in the total number of cells in the COX-2 group compared with controls was observed. In the Caov-3 cells, a similar decrease ($P < 0.05$) in the total number of cells in the acetaminophen group compared with controls was observed.

The expression of COX-1 and COX-2 in each cell line without any treatment as well as with each treatment was also evaluated. Figs. 3 and 4 show the expression of COX-1 and COX-2 in each cell line without any treatment via immunohistochemistry and Western blot analysis, respectively. Overall, the expression of COX-1 and COX-2 was lowest in the SKOV-3 cells and higher in the OVCAR-3 cells as observed through immunohistochemistry. Overall, COX-1 and COX-2 was expressed in each cell line. SKOV-3 cells expressed COX-1 and COX-2 relatively lower when compared with Caov-3 and OVCAR-3 cells as observed through immunohistochemistry. As shown in Fig. 3, the Caov-3 and OVCAR-3 cells suggest relatively more intense staining patterns for COX-1 and COX-2 in the cytoplasm and nucleus, although the intensity of the staining pattern differences is subtle. To clarify the COX enzyme expression patterns in the cancer cell lines, Western blots were performed with each cell line for COX enzyme expression. Our

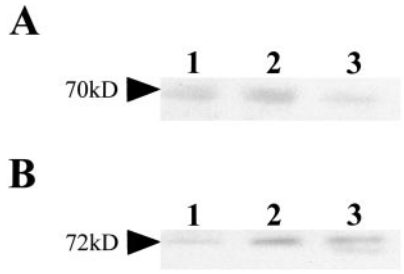

Fig. 4 Western blot analysis of COX-1 (*A*) and COX-2 (*B*) in cell lysates of untreated cultures of Caov-3, OVCAR-3, and SKOV-3 cells. A band at M_r 70,000 indicates protein expression of COX-1 and a band at M_r 72,000 indicates protein expression of COX-2.

immunohistochemical findings were consistent with Western blot analysis as shown in Fig. 4. A band at 70 kDa was observed in each cell line when the COX-1 antibody was used. A stronger band at M_r 70,000 (COX-1) was observed in the OVCAR-3 cell lysate. After probing each protein lysate with the COX-2 antibody, a band at M_r 72,000 was evident in each cell line. A lighter band was observed at M_r 72,000 (COX-2) in the SKOV-3 cells, whereas a stronger band at M_r 72,000 was observed in the OVCAR-3 cells. The expression of COX-1 and COX-2 was also determined via immunohistochemistry for each cell line after exposure to ASA, acetaminophen, and the COX-2 inhibitor at a concentration of 10^{-4} M. Overall, the immunohistochemistry scores for each treatment in each cell line did not show any striking differences compared with controls, although a slight elevation in the expression of COX-1 in the cytoplasm and nucleus of Caov-3 cells was observed after treatment with acetaminophen compared with controls.

The expression of erbB-2 was also determined via immunohistochemistry in each cell line treated with the same concentration of ASA, acetaminophen, and the COX-2 inhibitor. Changes in expression were not observed in the Caov-3 and OVCAR-3 cells with any of the treatments compared with controls. However, when the SKOV-3 cells were treated with ASA, differences in the expression of erbB-2 with varying concentrations were observed. With a higher concentration of aspirin (5×10^{-3} M), the expression of erbB-2 decreased within the cytoplasm and membrane of cells. Differences in the intensity and extent of staining of erbB-2 were not observed with higher concentrations of acetaminophen (5×10^{-3} M). However, a change in morphology of the cells was observed with higher concentrations of acetaminophen such that the cells had multiple elongated processes.

Although cultured tumor cells are useful for proof of principle experiments, these cells do not, at the time, mirror the behavior of primary ovarian cancer cells. Therefore, to demonstrate the cytotoxic effect of COX-2 inhibitors on ovarian cancer more specifically, we assessed the number of apoptotic bodies in the tumor cells of tissues treated with the COX-2 inhibitor, medium alone, or the vehicle (1% ethanol) at three time periods. We observed a greater number of apoptotic bodies in the tumor cells of tissues treated with the COX-2 inhibitor compared with tissues treated with medium alone or the vehicle at each time period. Fig. 5 is a representative photograph showing the presence of apoptotic bodies in human ovarian tumor tissue exposed to medium alone, 1% ethanol (vehicle), and the COX-2 inhibitor for 40 h.

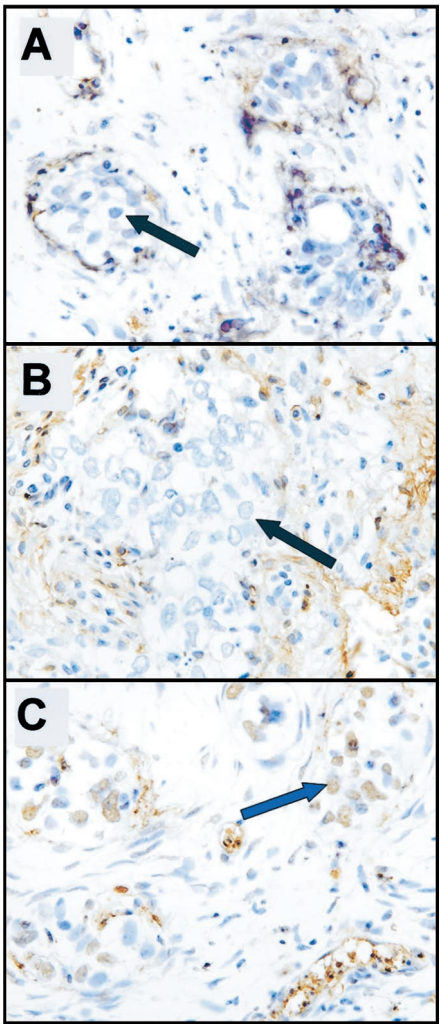

Fig. 5 Representative photographs of primary human ovarian carcinoma tissue exposed to medium alone (*A*), vehicle (1% ethanol; *B*), or the COX-2 inhibitor (*C*) for 40 h and then assessed for apoptosis using the TUNEL assay. *Blue arrow* points to a tumor cell stained positive for undergoing apoptosis. *Black arrows* point to tumor cells stained negative for undergoing apoptosis.

DISCUSSION

The purpose of these studies was to explore the potential utility of NSAIDs as chemopreventive agents in ovarian carcinoma. The use of aspirin and other NSAIDs has been associated with lower risk of colorectal cancer in epidemiological and experimental studies (20–24). These studies led Cramer *et al.* (9) to examine the relationship of over-the-counter analgesics and the risk of ovarian carcinoma in a population-based case-control study. In this study, 563 women known to have epithelial ovarian carcinoma (identified from a cancer registry) were compared with 523 controls (identified by random digit dialing). The specific agents that were addressed in this study were ibuprofen, aspirin, and paracetamol (acetaminophen). The odds ratio for risk of ovarian cancer and aspirin use was 0.75 (95% CI 0.52–1.10), for ibuprofen was 1.03 (95% CI 0.64–1.64), and for paracetamol was 0.52 (95% CI 0.31–0.86). The significantly

decreased risk of ovarian cancer attributable to use of paracetamol was maintained when controlled for potential confounding factors such as parity and oral contraceptive exposure. Similarly, in a recent report by Akhmedkhanov *et al.* (10), women who reported aspirin use in at least two follow-up questionnaires had a significantly lower incidence of epithelial ovarian cancer compared with women who never reported aspirin use (RR = 0.30; 95% CI, 0.09–0.99).

Consistent with the biological effects of NSAIDs on reduction of ovarian cancer incidence suggested in the epidemiological study by Cramer *et al.* (9), the current investigation provides *in vitro* evidence of direct growth inhibitory effects of these agents, particularly the COX-2 inhibitor, across all of the cell lines tested. Moreover, this growth inhibition of ovarian cancer cells occurs in a dose-dependent fashion. However, with the relatively high concentrations of NSAIDs at which growth inhibition was observed beyond therapeutic ranges of all of the agents because of the limitations of *in vitro* testing, we could only test these agents *in vitro* for up to 4 days, and we noticed reduced effects on testing for 2 days. The highest dose of the COX-2 inhibitor that we tested was 10^{-4} M. The maximum tolerated dose of the COX-2 inhibitor is considered to be ~1200 mg, and the chemopreventive dose of the COX-2 inhibitor is considered to be 200 mg, which approximates to 1.15×10^{-4} M and 1.9×10^{-5} M, respectively. The results suggest that long-term exposures may have inhibitory effects at much lower concentrations. The reduction of Ki-67 expression and mitotic activity observed in each cell line treated with the COX-2 inhibitor is also consistent with inhibitory effects on tumor cell proliferation by these agents. However the lack of a significant decrease in Ki-67 in Caov-3 cells and the moderate declines in OVCAR-3 and SKOV-3 cells suggest that mechanisms other than inhibition of the cell cycle are likely involved in the decrease in viable cells at 96 h.

Whereas the mechanism of NSAID-mediated inhibition of ovarian cancer cell growth has not been extensively investigated, the anticancer property of the NSAIDs is thought to reside in the ability to block the COX enzyme. Epidemiological studies have shown a 40–50% reduction in mortality from colorectal cancer in persons taking NSAIDs (25). These observational results have suggested that the use of NSAIDs may cause early disruption of the adenoma to carcinoma sequence. One of the mechanisms by which NSAIDs are thought to inhibit colon carcinogenesis is through the inhibition of prostaglandin production by COX isoenzymes (COX-1 and COX-2; Ref. 23). Nonspecific inhibition of COX enzymes is considered to be an important inhibitory effect of aspirin on the growth of tumor cells (25). The identification of agents that selectively inhibit COX-2 is important clinically, because simultaneous inhibition of COX-1 with classical NSAIDs interferes with normal housekeeping functions of COX-1 resulting in serious side effects such as peptic ulcer disease (25). Moreover, the overexpression of COX-2 in epithelial cells inhibits apoptosis and increases the invasiveness of tumor cells (25). These observations have led investigators to evaluate selective COX-2 inhibitors as chemopreventive agents against the development of adenocarcinoma of the colon. COX is a key enzyme in the conversion of arachidonic acid to prostaglandins (26). Moreover COX-2 can be expressed at high levels in human intestinal tumors (26). *In vitro* experiments have demonstrated significant growth inhibition of HCA-7 colon carcinoma cells by a selective COX-2 inhibitor (26). A selective COX-2 inhibitor decreased the size and number of colonies from HCA-7 cells starting at a dose of 10^{-5} M. We observed similar growth inhibition in the HT29 and WiDr colon cell lines.[5] Thus, the doses that caused inhibition of the growth of colon cancer cell lines were similar to that observed for ovarian cancer cell lines. In a murine model of azoxymethane induced colon tumors, the administration of a selective COX-2 inhibitor was found to inhibit both the incidence and multiplicity of colon tumors by 94 and 97%, respectively (24). Consideration must also be given to alternative pathways of tumor cell growth inhibition associated with COX inhibitors, particularly given the poor correlation between COX expression and the robust inhibition of ovarian cancer cell growth associated with the COX-2 inhibitor identified in this study. Li *et al.* (27) have demonstrated apoptosis in colon cancer cells using NS398 induced by activation of the caspase-9, caspase-3, and poly(ADP-ribose) polymerase components of the cytochrome *c* pathway. Moreover, Shureiqi *et al.* (28) have demonstrated that NSAIDs (NS-398) can induce apoptosis in colon cancer cells via up-regulation of 15 LOX-1 in the absence of COX-2. Finally, Zhang and DuBois (29) have suggested that regulation of Par-4 contributes to the proapoptotic effects of high-dose COX inhibitors by serving as a downstream mediator leading to initiation of programmed cell death.

Whereas the anticancer mechanism invoked most often for NSAIDs relates to inhibition of COX, acetaminophen is a poor inhibitor of COX, has only weak anti-inflammatory activity, and has not been demonstrated to have a protective effect on colorectal carcinoma (9). however However, interesting evidence does exist of an antigonadotropic effect of acetaminophen in animal studies. Acetaminophen has a phenol ring similar to estradiol and an acetyl group similar to progesterone, indicating a potential sex steroid antagonist property (9). Evidence of this antigonadotropic property was suggested by toxicological studies demonstrating uterine, ovarian, and testicular atrophy in rats fed acetaminophen at 25,000 parts per million (30). Cramer *et al.* (9) have postulated that the ovarian suppression induced by acetaminophen in mice may be secondary to a consequence of its metabolism. Specifically, acetaminophen is metabolized in the liver by conjugation to sulfate or glucuronide in a reaction requiring glutathione (31). Because glutathione is required both for the release of FSH and its receptor binding, glutathione depletion from acetaminophen metabolism could reduce the effective concentration of FSH (9). Shiffenbauer *et al.* (32) have demonstrated that loss of ovarian function with a concomitant rise in FSH levels promotes tumor angiogenesis. Using murine models, these authors demonstrated enhanced tumor growth in oophorectamized animals with associated FSH elevation. Moreover, tumor growth could be induced by administration of gonadotropins. Using magnetic resonance imaging, oophorectomy was demonstrated to enhance significantly ($P < 0.02$) neovascularization in implanted tumor spheroids. In a related finding, gonadotropin stimulation resulted in increased expression of vascular endothelial growth factor. The authors sug-

[5] Unpublished observation.

gested that therapy aimed at lowering circulating levels of gonadotropins might prolong remission in ovarian cancer by extending tumor dormancy.

These results should stimulate continued research into the potential of NSAIDs to provide chemopreventive effects in the setting of ovarian carcinoma. To this end, several limitations recognized in this study should be addressed with additional investigation. The use of ovarian cancer cell lines is a poor surrogate for chemoprevention models. These agents will need to exert a protective effect on normal tissues "at risk" and, as such, NSAID effects should be studied in normal ovarian lining, fallopian tube, and peritoneal tissues. Identification of biomarker alterations induced in these tissues by NSAIDs that correlate with a "protective effect" is critical to translational efforts in chemoprevention. Additionally, whereas the doses used to obtain growth inhibitory effects in this study are supra-physiological, the long term protective effects of chronic low dose administration of these agents needs to be confirmed. Therefore, it is anticipated that this report and others will stimulate investigation in this neglected area of study.

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Clin Cancer Res 2002;8:202-209.