ABSTRACT

Eicosanoids modulate the interaction of tumor cells with various host components in cancer metastasis. Their synthesis involves the release of arachidonic acid (AA) from cellular phospholipids by phospholipase A2 (PLA2), followed by metabolism by cyclooxygenases (COXs) and lipooxygenases (LOXs). This study aimed to identify the pathway(s) of AA metabolism that are required for the invasion of prostate tumor cells. DU-145 and PC-3 human prostate cancer cell lines were used to test the effect of inhibitors of PLA2, COX, or LOX on the invasion of prostate tumor cells through Matrigel in vitro using the Boyden chamber assay and fibroblast-conditioned medium as the chemoattractant. We used nontoxic doses that did not inhibit simple cell motility and did not decrease clonogenic survival. All of the inhibitors caused a significant reduction in AA release from treated cells compared with control cells, which indicated that the treatments were biochemically active. Invasion through Matrigel was inhibited by the PLA2 inhibitor 4-bromophenacyl bromide (4-BPB), the general COX inhibitor ibuprofen (IB), and the highly selective COX-2 inhibitor NS398. Inhibition of cell invasiveness by 4-BPB (1.0 \( \mu \text{M} \)), IB (10.0 \( \mu \text{M} \)), and NS398 (10.0 \( \mu \text{M} \)) was reversed by the addition of prostaglandin \( \text{E}_2 \) (PGE\(_2\)), PGE\(_2\) alone, however, did not stimulate invasiveness, which suggests that its production is necessary for rendering the cells invasive-permissive but not sufficient for inducing invasiveness. In contrast, we found no significant inhibition of invasion of prostate tumor cells treated with esculetin (1.0 \( \mu \text{M} \)) or nordihydroguaiaretic acid (1.0 \( \mu \text{M} \)), which are specific inhibitors of LOX. We also tested the effect of 4-BPB, IB, NS398, and esculetin on the secretion of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs), as key enzymes in the proteolysis of Matrigel during invasion, using gelatin zymograms and Western blots. Cells that received 4-BPB, IB, or NS398, but not esculetin showed a significant reduction in the levels of proMMP-2, MMP-9, and proMMP-9 in the culture medium. DU-145 cells did not secrete TIMP-1, and the drugs did not alter the secretion of TIMP-2. This work highlights the role played by COX in disturbing the balance between MMPs and TIMPs in prostate cancer cells, and it points to the potential use of COX inhibitors, especially COX-2 selective inhibitors, in the prevention and therapy of prostate cancer invasion.

INTRODUCTION

The prostate is the most common site of cancer in men, accounting for 29% of all of the estimated new cancer cases diagnosed in American males. It is the second leading cause of deaths for males, with one in every five men developing invasive cancer (1). If discovered in its early stages, prostate cancer can be successfully treated. However, it is usually fatal once the tumor cells invade outside the area of the gland, because of the unavailability of effective systemic therapies (2).

Tumor metastasis is a complex series of events in which metastatic cells migrate beyond tissue compartment boundaries and spread into different organs (3). Basement membranes are thin extracellular structures surrounding most epithelial tissues, nerves, muscles, and the linings of most blood vessels. They are composed of laminin, collagen IV, heparin sulfate proteoglycan, and entactin (4). Degradation of these basement membranes is a distinctive feature of invasive cells and has been the target of extensive research in an attempt to understand the signal transduction pathways involved in regulating this critical step of tumor invasion. These pathways may represent promising areas to direct the development of therapeutic agents that could prove useful in treating cancer.

The pattern of AA\(^3\) transformation in tumor cells has been shown to play a role in determining tumor cell invasiveness and metastases (5). AA is released from membrane phospholipids by PLA2. It is then metabolized into prostaglandins and thromboxanes via the COX system, and to leukotrienes and eicosatetraenoids via the LOX pathway. Products of both COX and LOX pathways that are secreted by tumor and/or host cells are thought to influence a variety of biological activities such as cell proliferation, cell movement, carcinogenesis, tumor promotion, and tumor metastasis (6, 7). Our current knowledge about the role of these key lipids in prostate cancer proliferation and invasion is limited. The rate of AA turnover in prostate tumor cells is 10-fold more than its rate in the surrounding normal cells (8). In addition, this increase in AA turnover was associated with an increase in PGE\(_2\) synthesis from labeled AA in malignant prostatic tissues (9). Gao et al. (10) established a correlation between 12-LOX mRNA expression and advanced prostate cancer stage and poor differentiation of human prostate cancer. In PC-3 prostate epithelial tumor cells, cytosolic PLA2 is constitutively activated by unknown mechanisms (11), and LNCaP human prostate cancer cells undergo apoptosis when they are deprived of AA and its metabolites (12). NSAIDs are among the most commonly prescribed drugs. Although they are primarily prescribed for controlling inflammation, some of these drugs have demonstrated anticancer activity in animal and human studies (13). Several lines of evidence from animal work and clinical studies confirmed their role in the inhibition of colon cancer initiation and progression (14). In addition, a recent population-based case-control study indicated that men who regularly took NSAIDs have a reduced relative risk of developing prostate cancer (15). IB, a nonspecific COX inhibitor, has been shown to significantly lower the number of metastatic lung tumors in mice receiving a s.c. injection of a metastatic mammary C3L5 carcinoma cell line, as well as to inhibit local tumor growth (16). In addition, the growth of primary esophageal tumors was delayed in animals treated with COX inhibitors such as aspirin, flurbiprofen, and indomethacin (17). Recently, selective inhibitors of the COX-2 isoform have attracted considerable attention because of their ability to selectively inhibit the inducible COX-2 isoform while allowing COX-1 to perform its “housekeeping” functions. This significantly reduces the gastrointestinal and renal side effects caused by systemic NSAIDs.

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Inhibitors of Prostaglandin Synthesis Inhibit Human Prostate Tumor Cell Invasiveness and Reduce the Release of Matrix Metalloproteinases\(^1\)

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[3] The abbreviations used are: AA, arachidonic acid; PLA2, phospholipase A2; COX, cyclooxygenase; LOX, lipooxygenase; PG, prostaglandin; PGE\(_2\), prostaglandin \( \text{E}_2 \); NSAID, nonsteroidal anti-inflammatory drug; 4-BPB, 4-bromophenacyl bromide; IB, ibuprofen; ES, esculetin; NDGA, nordihydroguaiaretic acid; FC, fibroblast-conditioned medium; MMP, matrix metalloproteinase; TIMP, tissue inhibitor(s) of metalloproteinase; SFM, serum-free medium; FP, peroxisome proliferator-activated receptor; BM, basement membrane; 12-HETE, 12(\( \alpha \)-hydroxyeicosatetraenoic acid.

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effects of NSAIDs, which have greatly limited the wide use of these drugs as chemopreventive agents (18).

The mechanism by which these drugs inhibit tumor growth and progression is unclear, and our knowledge about their potential in prostate cancer therapy is far from adequate. Therefore, we sought to identify the effect of the various agents that disrupt AA metabolism on the ability of prostate cancer cells to invade through a reconstituted BM (Matrigel). We used 4-BBP as a PLA2 inhibitor, IB as a non-selective COX inhibitor, NS398 as a highly selective COX-2 inhibitor, and ES and NDGA as LOX inhibitors. The effects of these drugs on the survival and motility of prostate cancer cells were also assessed. In addition, to investigate the mechanism by which PLA2 and COX inhibitors inhibit invasion, we studied their effect on the FCM-induced secretion of MMPs/TIMPs from DU-145 cells.

MATERIALS AND METHODS

Cell Culture. DU-145 (19) and PC-3 (20) prostate epithelial tumor cell lines and mouse fibroblasts (NIH/3T3) were obtained from the American Type Culture Collection (Rockville, MD). The cell lines were maintained in monolayer culture at 37°C under an atmosphere of 5% CO2 in MEM with Earle’s salts for DU-145 and α MEM for PC-3. NIH/3T3 fibroblasts were cultured in DMEM. All of the media were supplemented with 10% fetal bovine serum, penicillin (100 units/ml) and streptomycin (100 μg/ml). Cells were fed with fresh medium every 3rd day and were passaged routinely at a confluence of ~80%.

Chemicals. DMSO, 4-BBP, IB, ES, NDGA, indomethacin and quinacrine were obtained from Sigma (St. Louis, MO). NS398 was obtained from Cayman Chemicals (Ann Arbor, MI). Mouse antihuman monoclonal antibodies for MMP-2 (MAB3308), MMP-9 (MAB3309), TIMP-1 (MAB3301) and TIMP-2 (MAB3310) were obtained from Chemicon International Inc. (Temecula, CA).

Invasion assay. Matrigel invasion assays were performed as described previously (21). Briefly, tumor cells were treated with different concentrations of each drug, whereas control cells received the vehicle only. After 12–18 h of treatment, cells were removed by gently scraping with a cell scraper or by trypsinizing, and their in vitro invasiveness was tested by the Boyden chamber invasion assay. FCM was used as a chemoattractant, and was obtained by incubating subconfluent mouse fibroblasts (NIH/3T3) for 24 h in SFM containing 0.5 μg/ml BSA. To assess stimulated invasion, FCM (220 μg/ml) was placed in the lower compartment of the Boyden chamber (Neuro Probe Inc., Gaithersburg, MD; 8 mm in diameter). For analysis of basal invasion, SFM was placed in the lower chamber. Matrigel (Collaborative Biomedical Products, Bedford, MA; 10 mg/ml) was diluted to 25 μg/ml and applied to 12 mm 6-micron pore size polyvinylpyrrolidone-free polycarbonate membrane filters (Neuro Probe Inc.). Filters were then air-dried overnight in a laminar flow hood. Prostate tumor cells in SFM were added to the upper compartment (3.0 × 104 cells/well), and the chambers were incubated for 6 h at 37°C. Invasiveness was assayed in triplicate for each condition, in at least three independent experiments. At the end of the incubation period, the Matrigel and the cells on the upper surface of the filter were completely removed by wiping with a moist cotton swab. Cells that had invaded the filter and the cells on the upper surface of the filter were completely removed by wiping with a moist cotton swab. Cells that had invaded the Matrigel and had migrated through the filter and adhered to its lower surface were stained for 10 min with 0.5% crystal violet in 25% methanol, according to the method of Frandsen et al. (22). The filters were rinsed in distilled water until no additional stain leached and were air-dried overnight. The crystal violet was extracted from the invading cells by adding 900 μl of 0.1 M sodium citrate in 50% ethanol. Absorbance was measured spectrophotometrically at 585 nm using Spectronic GENESYS 5 (Milton Roy, Rochester, NY).

Clonogenicity Assay. To assess the effect of the inhibitors of AA metabolism on the ability of tumor cells to divide, tumor cells at ~80% confluency were treated for 12–18 h with SFM containing the highest dose of drugs used in the invasion assay. Cells were then trypsinized and seeded in triplicate at 200 cells/60-mm plate for DU-145 cells and 400 cells/60-mm plate for PC-3 cells. After 12 days at 37°C in a humidified 5% CO2 atmosphere, interrupted by feeding cells with a fresh medium every 3rd day, colonies were stained with crystal violet and counted. Cloning efficiencies were scored and comparisons were made between vehicle-treated cells and those that received the drugs.

Motility Assay. To determine the effect of the inhibitors of AA metabolism on cell motility, treated cells were seeded into Boyden chambers on membrane filters that were not coated with Matrigel. Migration in the absence or presence of FCM in the bottom chamber was measured as described in the invasion assay.

AA Release. [14C]AA (specific activity, 56 mCi/mmol), obtained from Amersham (Arlington Heights, IL) was used to measure AA release stimulated by FCM from DU-145 prostate epithelial tumor cells that were treated with inhibitors of AA metabolism according to the method of Leyton et al. (23). Cells were labeled with preincubation with 1 μCi of [14C]AA per 0.75 × 106 cell in 2 ml of SFM for 18 h at 37°C. Excess radioactivity was washed away by adding 5 ml of 0.2% BSA in SFM and repeating the wash three times. Drugs were added in a final volume of 5 ml, and incubated for an additional 6 h. A time course evaluation of [14C]AA release was recorded on stimulating vehicle-treated and drug-treated cells with FCM, by sampling 50-μl aliquots from each flask at 0, 5, 10, 20, 30, 60, and 120 min. Radioactivity was measured as cpm on a scintillation counter.

Zymogels. Analysis of the levels of MMPs released into SFM by DU-145 cells under the induction of FCM was performed using SDS-polyacrylamide gel zymography (24). DU-145 cells (2 × 105 cell) were seeded in 25-mm cell culture inserts that have tissue culture-treated polyethylene terephthalate track-etched membrane with 1.0 μm pore size, and six inserts were placed in six-well tissue culture plates (Becton Dickinson, Bedford, MA). The cells were allowed to grow in complete MEM medium with Earle’s salts for 24 h, and then the plate was examined under a light microscope for contiguity of the cell monolayer formed on the insert membrane. The inserts were then washed with SFM, supplemented with 0.1% BSA, to both the well and the insert for 18 h. For the controls, cells in one insert were washed with PBS and treated with 10 μg/ml cycloheximide for 3 h, whereas cells in another insert were fixed using 4% paraformaldehyde in PBS for 10 min. FCM was added to each well, and 500 μl of SFM supplemented with 0.1% BSA was added to the insert. After incubation at 37°C for 24 h, the medium from each insert was collected and analyzed by zymography and Western blotting. Samples (10 μl) of media collected from the cell inserts that contained drug-treated or vehicle-treated cells were mixed 2:1 with sample loading buffer [0.25 M Tris-HCl (pH 6.8), 10% SDS, 4% sucrose, and 0.1% bromophenol blue]. Samples were loaded on 4% acrylamide Laemmli stacking gel on a Mini-Slab gel apparatus (Bio-Rad, Hercules, CA), and were electrophoresed (120 V) at 4°C on a 10% acrylamide-resolving minigel containing 0.1% gelatin (24). After electrophoresis for 3 h, the gels were soaked in 2.5% Trito X-100 on a shaker for 1 h, changing the solution after 30 min, to eliminate SDS. After overnight incubation in zymogen activation buffer [50 mM Tris-HCl (pH 7.5), containing 10 mM CaCl2] at 37°C, the gels were rinsed in distilled water in preparation for staining. The gels were stained for 2–3 h with PhastGel Blue R stain as described (25), after which clear bands of digested gelatin were clearly visible. The gels were briefly rinsed in distilled water and scanned by the Eagle Eye II still-video imaging system (Stratagene, La Jolla, CA).

Western Blotting. Samples (10 μl) of media collected from the cell inserts that had drug-treated or vehicle-treated cells were mixed with sample buffer supplemented with 10% β-mercaptoethanol in a 2:1 ratio. Samples were electrophoresed (120 V) on a 10% acrylamide resolving gel for 1.5 h when probing for TIMP-1 and TIMP-2, and for 3 h when probing for MMP-2 and MMP-9. The gel was then soaked in 1× transfer buffer for 15 min [25× transfer buffer contains 300 mM Tris base (pH 8.3) and 2.4 mM glycine]. Protein transfer was done by electroblotting onto polyvinylidyne difluoride membrane for 1 h at 30 V. Blots were incubated overnight in 10% nonfat dry milk solution in 1× wash buffer (0.3% Tween 20, 140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4). Then, the membrane was probed for 1 h with 10 μl of the mouse antihuman-monoclonal antibody (1:1000 dilution) for MMP-2, MMP-3, TIMP-1, or TIMP-2, in 10 ml of 10% milk solution made with wash buffer. Antimouse immunoglobulin horseradish peroxidase-linked whole antibody (4 μl, 1:2500 dilution) in 10 ml of wash buffer with 10% milk was added to the membrane and incubated for 1 h. Secondary antibody was detected using enhanced chemiluminescence followed by autoradiography.
RESULTS

Effect of PLA₂ Inhibitor 4-BPB. Treatment of DU-145 and PC-3 prostate tumor cells with 0.01 μM, 0.1 μM, and 1.0 μM 4-BPB for 12–18 h resulted in a dose-dependent inhibition of FCM-stimulated invasiveness through Matrigel in both of the cell lines (Fig. 1). Inhibition of invasion with 1.0 μM was significant at P < 0.01 for DU-145 and PC-3 with ≤70% inhibition compared with vehicle-treated cells. A similar pattern of dose-dependent inhibition of invasion (significant at P < 0.01) was seen on treatment of DU-145 cells with quinacrine, another PLA₂ inhibitor (data not shown), with ≤60% inhibition of invasion when cells were treated with 1.0 μM quinacrine. Neither drug affected the basal invasion of cells through Matrigel when SFM was placed in the bottom compartment of the Boyden chamber (data not shown).

Effect of COX Inhibitors. We treated DU-145 and PC-3 prostate cancer cells with IB as an inhibitor of the COX pathway of AA metabolism. IB inhibits COX-1 and COX-2 isoforms. Control cells that were treated with only DMSO readily penetrated through Matrigel under induction by FCM. On the other hand, cells pretreated with 0.1 μM, 1.0 μM, or 10.0 μM of IB showed a significantly lower level of induced invasion at P < 0.01 using Student’s t test (Fig. 2). IB (10.0 μM) caused 80% inhibition of invasion in DU-145 cells and about 65% inhibition of invasion in PC-3 cells. A similar pattern of dose-dependent inhibition of invasion was seen on treatment of cells with indomethacin (data not shown), which at 10.0 μM caused ≤72% inhibition of invasion (P < 0.01). When DU-145 cells were treated with increasing concentrations of a highly selective COX-2 inhibitor, NS398, the drug caused dose-dependent inhibition of cell invasiveness through Matrigel. Invasiveness of NS398-treated cells was significantly different from that in control cells at P < 0.01 using Student’s t test. NS398 caused ≤40% inhibition of invasion at 0.1 μM and ~85% inhibition of invasion at the 10.0-μM dose (Fig. 3).

Effect of PGE₂. In these experiments, 4.0 ng/ml PGE₂ was added to both compartments of the Boyden chamber during the invasion assay. PGE₂ reversed the anti-invasive effect of 4-BPB (Fig. 4A), IB (Fig. 4B), and NS398 (Fig. 4C). PGE₂ given alone did not significantly influence the FCM-induced invasiveness of DU-145 and PC-3 prostate tumor cell lines (Fig. 4A, B, and C), and did not stimulate the basal invasiveness of DU-145 cells in the absence of FCM (Fig. 4D), even at high concentrations (4 μg/ml PGE₂).

Effect of LOX Inhibitors. When ES or NDGA was added to the cells in culture 12 h prior to the assay, the ability of the cells to invade through Matrigel was not reduced as was seen with the PLA₂ and COX inhibitors. No significant inhibition of invasion was seen when cells were treated with 0.01 μM, 0.1 μM, or 1.0 μM of ES (Fig. 5) or NDGA at the same concentrations (Fig. 5). Neither drug affected basal invasion (data not shown). Higher doses of ES and NDGA (5.0 and 10.0 μM) showed evidence of cytotoxicity by decreasing cell survival in a clonogenic assay by about 25–45% (data not shown). Therefore, the highest dose of ES and NDGA that was used in subsequent experiments was 1.0 μM, which did not affect cell survival even after 24 h of treatment.

Effect of NSAIDs on FCM-induced AA Release. To document that all of the drugs were interfering with AA metabolism, DU-145

Fig. 1. The effect of 4-BPB on the invasion of DU-145 and PC-3 prostate tumor cells through Matrigel. Cells were treated with 0.01 μM, 0.1 μM, or 1.0 μM of 4-BPB for 12–18 h. Control cells received 0.1% DMSO only. Inhibition was quantified by subtracting basal invasion from FCM-stimulated invasion and expressed as percentage of control vehicle-treated cells. Data represent the mean ± SE of at least three independent experiments. Results were statistically significant (*, P < 0.05; **, P < 0.01) using Student’s t test.

Fig. 2. The effect of IB on the invasion of DU-145 and PC-3 prostate tumor cells through Matrigel. Cells were treated with 0.1 μM, 1.0 μM, or 10.0 μM of IB for 12–18 h. Control cells received 0.1% DMSO only. Inhibition was quantified by subtracting basal invasion from FCM-stimulated invasion and was expressed as percentage of control vehicle-treated cells. Data represent the mean ± SE of at least three independent experiments. Results were significantly different from control (*, P < 0.05; **, P < 0.01) using Student’s t test.

Fig. 3. The effect of the selective COX-2 inhibitor NS398 on the invasion of DU-145 prostate tumor cells through Matrigel. Cells were treated with 0.1 μM, 1.0 μM, or 10.0 μM of NS398 for 12–18 h. Control cells received 0.1% DMSO only. Inhibition was quantified by subtracting basal invasion from FCM-stimulated invasion and was expressed as percentage of control vehicle-treated cells. Data represent the mean ± SE of at least three independent experiments. Results were significantly different from control (*, P < 0.01) using Student’s t test.

Fig. 4. The effect of 4-BPB on the invasion of DU-145 and PC-3 prostate tumor cells through Matrigel. Cells were treated with 0.01 μM, 0.1 μM, or 1.0 μM of 4-BPB for 12–18 h. Control cells received 0.1% DMSO only. Inhibition was quantified by subtracting basal invasion from FCM-stimulated invasion and expressed as percentage of control vehicle-treated cells. Data represent the mean ± SE of at least three independent experiments. Results were statistically significant (*, P < 0.05; **, P < 0.01) using Student’s t test.

Statistical Analysis. Each experiment was done in triplicate, and repeated at least three times. Comparisons between the test group and controls in this study were evaluated using Student’s t test.
prostate epithelial tumor cells were labeled with $[^{14}C]AA$ as described in “Materials and Methods.” This was followed by stimulation of the release of radioactive AA and/or its metabolites from cells by treatment with FCM; this release was compared with the amount of release between cells that were pretreated with inhibitors of AA metabolism and cells that received the vehicle only. FCM induced a typical biphasic release pattern of $[^{14}C]AA$ from the cells after induction (Fig. 6). Pretreatment with the PLA$_2$ inhibitor (4-BPB), COX inhibitors (IB and NS398), and LOX inhibitor (ES) markedly reduced FCM-stimulated release of $[^{14}C]AA$ from DU-145 cells (Fig. 6). The level of radioactive AA released from treated cells was significantly lower than the level secreted from control cells at $P < 0.05$ using Student’s $t$ test.

**Effect of NSAIDs on the Survival of Prostate Cancer Cells.** We performed colony-forming assays on cells that were pretreated with 4-BPB, IB, ES and NDGA to assess the cytotoxicity caused by these drugs at the highest dose used in the invasion assay. As seen in Fig. 7, the highest dose of all of the drugs used in the invasion assay had no significant effect on the colony-forming ability of either cell line. The vehicle DMSO (0.1%) was not toxic to the prostate tumor cells and had no effect on the ability of tumor cells to divide. These results confirm that the anti-invasive effect of the various inhibitors of AA metabolism we studied was not due to drug toxicity.

**Effect of NSAIDs on the Motility of Prostate Cancer Cells.** Motility was examined because it is an important component of the invasion process, and it serves as a sensitive indicator of nonspecific drug toxicity at the time of the invasion assay. To determine whether the anti-invasive activity of these drugs was attributable to their effect on cell motility, cellular chemotaxis of DU-145 and PC-3 toward FCM was tested using Boyden chambers that were prepared with uncoated filters (no Matrigel). None of the drugs tested, at the highest concentration used in the invasion assay, reduced the ability of prostate tumor cells to cross the filters toward FCM (Fig. 8). Basal migration, with SFM in the lower chamber, was minimal in both untreated and treated cells (data not shown).
Effect of NSAIDs on the Secretion of MMPs from Prostate Cancer Cells. Because MMPs and their inhibitors are known to bind to components of Matrigel such as collagen and laminin, a Matrigel-free cell insert system was developed as described in “Materials and Methods.” After treating DU-145 cells with 1.0 μM 4-BPB, 10.0 μM IB, 10.0 μM NS398, or 1.0 μM ES, cells were induced to secrete MMPs and/or their inhibitors into SFM by adding FCM to the well. Because the FCM itself might contain MMPs and TIMPs, it was necessary to account for the amounts of MMPs and/or TIMPs that diffused from FCM through the cell insert membrane. We did this by analyzing samples of SFM collected from paraformaldehyde-fixed DU-145 cells, as well as living cells that were pretreated with cycloheximide (10.0 μg/ml) for 3 h before induction with FCM, to inhibit their total protein synthesis. FCM was rich in MMPs and contributed about 30–50% of the proteolytic activity seen in samples (Figs. 9 and 10).

The activity of MMPs in DU-145 cells was first investigated in total protein extractions of cell lysates. Gelatin-digested bands were barely detectable on the zymogram, despite loading 300 μg of protein per lane (data not shown). Next, an analysis of MMP activity secreted from cells was carried out by loading 10 μl of the SFM on zymogels as described in “Materials and Methods.” As shown in Fig. 9, three bands appeared on the zymogram, which run at Mr 92,000, 74,000, and 72,000. These bands correspond to proMMP-9, proMMP-2, and MMP-2, respectively. In Fig. 9, Lanes 1 and 2 represent the proteases that diffused from the FCM in the well through the fixed cells (Lane 1) or the cycloheximide-treated cells (Lane 2) to the medium in the cell insert. These two bands account for the maximum contribution of MMPs in the FCM to the total MMPs in the insert. Lane 3 represents a sample from the medium that was collected after 24 h from vehicle-treated control cells; the difference between band density seen from samples in Lanes 1 and 2 compared with Lane 3 represents the protease activity actually secreted from the DU-145 cell monolayer in the cell insert under induction by FCM.

Samples of media collected from DU-145 cells that were treated with 1.0 μM 4-BPB (Fig. 9, Lane 4), 10.0 μM IB (Fig. 9, Lane 5), or 10.0 μM NS398 (Fig. 9, Lane 6) showed a significantly decreased
Methods.

Electrophoresed on gelatin containing acrylamide gels, as described in "Materials and Methods." Lanes 1, paraformaldehyde-fixed cells; Lane 2, cycloheximide-treated (10.0 μg/ml) cells; Lane 3, DMSO-treated cells; Lane 4, 4-BPB-treated (1.0 μM) cells; Lane 5, IB-treated (10.0 μM) cells; Lane 6, NS398-treated (10.0 μM) cells; Lane 7, ES-treated (1.0 μM) cells. This gel is a representative of four independent experiments. * M marker at 126,000; M̄ M̄ marker at 73,000.

In this particular gel, ES (1.0 μM) seemed to cause a slight reduction in the secreted MMP activity (Fig. 9, Lane 7), but the results of four independent experiments were not significantly different from the control DMSO-treated cells after analysis by densitometry and Student’s t test at P < 0.05 (Fig. 10). Blocking COX activity in DU-145 cells using IB and NS398 caused significant reduction (P < 0.01) in the proteolytic activity of secreted proMMP-9, proMMP-2, and active MMP-2. It should be noted that the artificial activation of proMMP-2 and proMMP-9 in zymography is well documented (26, 27). Analysis of the bands by densitometry (Fig. 10) showed that when COX-2 was inhibited in DU-145 cells by NS398, the proteolytic activity in cell secretion was 50–70% less than that secreted by control DMSO-treated cells. Because zymography measures proteolytic activity and not the amount of proteins, we analyzed the FCM-stimulated cell secretion by Western blotting using MMP-2 monoclonal antibody that detects active MMP-2 as well as itszymogen precursor proMMP-2. The results as seen in Fig. 11 confirmed the results that we obtained from zymogel (Fig. 9) and indicated that the decreased proteolytic activity was attributable to reductions in the amounts of proMMP-2 and MMP-2 secreted into the culture medium of DU-145 cells that were treated with 4-BPB (Lane 4), IB (Lane 5), or NS398 (Lane 6). In addition, data from Western blotting with a monoclonal antibody to human MMP-9 were in agreement with the zymogel data and showed a reduction in the amount of proMMP-9 that was secreted from DU-145 cells treated with 4-BPB, IB, and NS398 (Fig. 11).

Effect of NSAIDs on the Secretion of TIMPs from Prostate Cancer Cells. Western blot analysis of samples of media that were collected from control and treated DU-145 cells indicated that this prostate tumor cell line does not secrete TIMP-1 (Fig. 12). On the other hand, TIMP-2 was detected by its monoclonal antibody (Fig. 12); however, the data indicated that the various inhibitors used to block PLA2, COX, and LOX did not affect the ability of DU-145 cells to secrete TIMP-2 into the medium.

DISCUSSION

This is the first study to examine the role of AA metabolism in human prostate cell invasion by using several inhibitors that block key enzymes in AA metabolism. Nontoxic concentrations of several PLA2 and COX inhibitors were able to reduce FCM-stimulated invasion of prostate tumor cells. Cells that were treated with one of the AA inhibitors and cells that received the vehicle DMSO were induced to produce MMPs by FCM. MMPs were secreted from cells into 500 μl of SFM for 24 h. SFM from different conditions were collected and analyzed by gelatin-SDS-PAGE. Samples (10 μl) from the media were electrophoresed on gelatin containing acrylamide gels, as described in “Materials and Methods.”

Lane 1, paraformaldehyde-fixed cells; Lane 2, cycloheximide-treated (10.0 μg/ml) cells; Lane 3, DMSO-treated cells; Lane 4, 4-BPB-treated (1.0 μM) cells; Lane 5, IB-treated (10.0 μM) cells; Lane 6, NS398-treated (10.0 μM) cells; Lane 7, ES-treated (1.0 μM) cells. This gel is a representative of four independent experiments. * M marker at 126,000; M̄ M̄ marker at 73,000.

Fig. 8. The effect of inhibitors of the AA metabolism on the motility of DU-145 and PC-3 prostate epithelial tumor cells. Cells were treated with 1.0 μM 4-BPB, 10.0 μM IB, 10.0 μM NS398, 1.0 μM ES, or 1.0 μM NDGA. Data represent mean ± SE of at least three independent experiments. * significantly different from control (P < 0.05) using Student's t test.

Fig. 9. Zymographic analysis of MMP expression in the culture media of DU-145 prostate tumor cells. Cells that were treated with one of the AA inhibitors and cells that received the vehicle DMSO were induced to produce MMPs by FCM. MMPs were secreted from cells into 500 μl of SFM for 24 h. SFM from different conditions were collected and analyzed by gelatin-SDS-PAGE. Samples (10 μl) from the media were electrophoresed on gelatin containing acrylamide gels, as described in “Materials and Methods.” Lane 1, paraformaldehyde-fixed cells; Lane 2, cycloheximide-treated (10.0 μg/ml) cells; Lane 3, DMSO-treated cells; Lane 4, 4-BPB-treated (1.0 μM) cells; Lane 5, IB-treated (10.0 μM) cells; Lane 6, NS398-treated (10.0 μM) cells; Lane 7, ES-treated (1.0 μM) cells. This gel is a representative of four independent experiments. * M marker at 126,000; M̄ M̄ marker at 73,000.

Fig. 10. Densitometric analysis of the secretion of MMPs from DU-145 cells. Cells were treated with 0.1% DMSO (control), 1.0 μM 4-BPB, 10.0 μM IB, 10.0 μM NS398, or 1.0 μM ES. After treatment, cells were induced to secrete MMPs into SFM by FCM for 24 h. Data represent the average of four independent zymograms. In each case, we averaged the MMP that was produced from fixed cells and from cells that were treated with 10.0 μg/ml cycloheximide (Lanes 1 and 2 in Fig. 9), and presented that as MMPs derived from FCM. Data is represented as percentage of control for (A) ProMMP-2 and MMP2 and (B) ProMMP-9. * significantly different from control (P < 0.05) using Student’s t test.
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prostate epithelial tumor cells by \( \leq 60-80\% \) of that seen in the control cells. This reduced invasion was not associated with reduced basal invasion, decreased cell motility, or drug toxicity. The results imply the involvement of PLA\(_2\) and COX in a specific invasion-associated signal cascade, which was induced by the presence of FCM in the lower compartment of the Boyden chamber.

Similar effects of NSAIDs on tumor cell growth and/or invasion were reported in colon cells (14, 28), mammary carcinoma cells (16, 29), mouse esophageal tumor cells (17), human urinary bladder carcinoma (30), mouse melanoma, and human fibrosarcoma cells (31). Furthermore, animal work confirmed the chemopreventive potential of NSAIDs in several types of cancer. In addition, a positive correlation was reported between the amount of PGs production and tumor invasiveness in the lung (32). Inhibition of PGs synthesis by indomethacin in undernourished patients with metastatic solid tumors prolonged their survival when compared with placebo treatment (33), and treatment of several kinds of solid tumors with indomethacin resulted in retardation of tumor growth and aggressiveness (34).

Prostate tumor cells exhibit AA turnover at a rate that is 10-fold more than its rate in the surrounding normal cells (8), and the main product of AA metabolism in prostate epithelial cells is PGE\(_2\) (39). Our data imply a role for COX-2 metabolites in prostate cancer progression toward the invasion of BM, because selective inhibition of this isoform was able to reduce in vitro invasiveness by \( \leq 85\% \). This inhibition was reversed by PGE\(_2\), implicating PGE\(_2\) as an important mediator in the degradation of BM by prostate tumor cells. Interestingly, PGE\(_2\) itself did not stimulate the invasiveness of prostate tumor cells. This, together with the invasion inhibition/reversal data, indicates that COX-2 metabolites are necessary “invasive-permissive” factors but are not sufficient to induce invasiveness by themselves. The effect of COX-2 inhibition of DU-145 prostate tumor cell invasion in vitro points to a very promising therapeutic target in prostate cancer prevention and treatment, in view of the great success COX-2 inhibitors are demonstrating in colon cancer prevention and treatment (35). In regard to COX-2 inhibition, PC-3 cells were excluded from the rest of the experiments based on studies that showed that PC-3 and LNCaP human prostate cancer cell lines did not express 12-LOX and COX-2 AA-metabolizing enzymes when examined using reverse transcription-PCR, whereas DU-145 cells had both enzymes (36).

In DU-145 and PC-3 prostate cancer cells, the inhibition of AA metabolism through PLA\(_2\) by 4-BPP and the inhibition of COX by IB and NS398 in DU-145 cells did not affect tumor cell clonogenicity, as assessed by the ability of cells to survive and form colonies after 24 h of treatment with inhibitors. This indicates that the anti-invasive effect exerted by these drugs was not attributable to cytotoxicity as suggested by several investigators (11, 12, 14, 37). In all of these reports, treating cells with the same inhibitors used in this study, yet at much higher concentrations (100 \( \mu M \) to 1.0 \( \mu M \)), induced apoptosis. The nontoxic concentrations used in this work support the conclusion that the anti-invasive effect of these drugs was not merely attributable to cell death, but rather involved specific reduction of the ability of tumor cells to invade through Matrigel. This is further strengthened by the lack of effect of the different inhibitors on simple cell motility, which indicates the absence of nonspecific toxicity during the invasion assay.

Our results suggest that the LOX pathway does not mediate prostate tumor cell invasiveness through Matrigel. Treatment of cells with 0.01 \( \mu M \), 0.1 \( \mu M \), or 1.0 \( \mu M \) ES or NDGA for 12–18 h before the invasion assay did not cause any significant reduction of invasion when compared with control vehicle-treated cells. On the other hand, 1.0 \( \mu M \) ES significantly reduced the release of AA and/or its metabolites from DU-145 cells that were stimulated by FCM, which indicates that the drug was effectively interfering with AA metabolism. Our results are qualitatively different from other reports, which suggest the involvement of the LOX pathway of AA metabolism in tumor growth and invasion in vitro (10, 31, 38, 39). One research group (31) reported a dose-dependent inhibition of invasion of mouse melanoma and human fibrosarcoma cells by 10.0 and 50.0 \( \mu M \) ES. They reported that these concentrations were not toxic to the cells after 24 h exposure, but their analysis was limited to trypsin blue dye exclusion. In our studies, concentrations exceeding 5.0 \( \mu M \) of ES caused a marked reduction in clonogenic cell survival. We used the highest concentration of ES that did not exhibit toxicity (1.0 \( \mu M \)), and it did not affect cell invasiveness. Leukotrienes and eicosatetraenoids have been implicated in prostate tumor cell invasion, specifically through the 12-LOX pathway of AA metabolism. Pharmacological doses of 12-HETE (0.1 \( \mu M \)) have been reported to augment the motility and invasiveness of AT2.1 metastatic rat prostate tumor cells through Matrigel-coated filters via selective activation of protein kinase Cc (39). That approach is fundamentally different from the one used here, which found that the inhibition of the synthesis of 12-HETE in prostate cancer cells by inhibitors of LOX did not affect their growth or invasiveness. We did not test the effect of adding any of the products of the LOX pathway on DU-145 and PC-3 cells invasiveness because we did not find any

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**Fig. 11.** Western blot analysis of MMPs expression in the culture media of DU-145 prostate tumor cells. Vehicle-treated control cells and cells that were treated with one of the inhibitors of AA metabolism for 12 h were incubated for 24 h in 500 \( \mu l \) of SFM and were induced to produce MMPs by FCM. The media were collected and analyzed by SDS-PAGE, followed by probing with mouse antihuman MMP-2 or MMP-9 monoclonal antibodies. Lane 1, fixed cells; Lane 2, cycloheximide-treated cells; Lane 3, DMSO-treated cells; Lane 4, 4-BPP-treated cells; Lane 5, IB-treated cells; Lane 6, NS398-treated cells; Lane 7, ES-treated cells.

**Fig. 12.** Western blot analysis of TIMPs expression in the culture media of DU-145 prostate tumor cells. Vehicle-treated control cells and cells that were treated for 12 h with one of the inhibitors of AA metabolism were incubated in 500 \( \mu l \) of SFM for 24 h and were induced by FCM to produce MMP/TIMP. The media were collected and analyzed by SDS-PAGE, followed by probing with mouse antihuman TIMP-1 or TIMP-2 monoclonal antibodies. Lane 1, fixed cells; Lane 2, cycloheximide-treated cells; Lane 3, DMSO-treated cells; Lane 4, 4-BPP-treated cells; Lane 5, IB-treated cells; Lane 6, NS398-treated cells; Lane 7, ES-treated cells.
inhibitory effect on invasiveness by LOX inhibitors, ES and NDGA. Nevertheless, the lack of effect of ES on the invasiveness of these cells, whereas clearly interfering with AA metabolism and release, indicates that metabolites produced by AA through the LOX pathway are not essential for the ability of DU-145 cells to invade the BM.

In cancer, the proteolytic activity of any tumor cell is determined by the ratio of proteases to their inhibitors, and this activity has been repeatedly associated with the metastatic potential of tumor cells. To investigate the mechanism by which PLA₂ and COX inhibitors inhibit invasion, we studied their effect on the secretion and activity of MMPs/TIMPs from DU-145 cells under the induction by FCM. Gelatin zymography of MMPs and densitometric analysis revealed that the release of proMMP-2 and proMMP-9, as well as the levels of active MMP-2 in the culture medium of DU-145 cells, were reduced when cells were treated by 4-BPB (1.0 μM), IB (10.0 μM), and NS398 (10.0 μM). The human prostate cancer cells DU-145 did not secrete TIMP-1 in the growth medium, and treatment with 4-BPB, IB, NS398, or ES did not affect TIMP-2 production. ES, as a general LOX inhibitor did not cause a statistically significant reduction in the amount of MMPs secreted from DU-145 cells (Fig. 10). This result was in agreement with the data from the invasion assay, in which ES did not inhibit invasion.

In all of these experiments, the cells were induced to produce MMPs and/or TIMPs by the presence of FCM that itself contains MMPs. FCM contributed to about 30–50% of the total gelatin-degrading activity of the samples in the Matrigel-free system that we used. Such a system was necessary because MMPs are known to bind to components of the Matrigel, making it difficult to analyze the amount of MMPs secreted into the medium in the presence of Matrigel. In this assay, the diffusion of MMPs from the well to the insert occurred through a monolayer of DU-145 cells and occurred during a period of 24 h. In contrast, in the Boyden chamber invasion assay, MMPs in the FCM would need to diffuse across a Matrigel barrier in only 6 h. Thus there is likely to be a lower rate of diffusion, and hence a lower contribution of FCM-MMPs to the total proteolytic activity in the invasion assay. Nevertheless, it has been documented that proteinases are part of the “cross-talk” between epithelial cells and adjacent stromal cells (40–43). Therefore, the contribution of the FCM-MMPs to the total proteolytic activity in the invasion assay may actually resemble part of the stromal contribution to prostate cancer progression and invasion in vivo.

MMPs are a family of nine or more highly homologous Zn⁺⁺ endopeptidases that collectively cleave the constituents of extracellular matrix (44). In one study, metabolites of COX and LOX attenuated the production of MMP-2 in human fibrosarcoma and murine melanoma cells (31), and their contribution to prostate cancer progression is well documented (45, 46). In this study, inhibitors of both PLA₂ and COX were able to reduce the production of proMMP-2 and active MMP-2 from prostate cancer cells. Stearns and Stearns (46) showed increasing levels of active MMP-2 as the Gleason grade of the prostate cancer increased, which indicated an inverse relationship between differentiated state and enzyme level. Furthermore, DU-145 cells produced a considerable amount of proMMP-9 in its M₉₀,200 form in this study, in synchrony with another study, which showed that proMMP-9 was exclusively expressed by malignant prostatic tissue, and in particular by tumors that exhibited the aggressive and metastatic phenotype (47). IB and NS398 reduced the levels of both latent and active forms of MMP-2 in the culture medium from treated cells. However, it is not clear whether these drugs are able to inhibit the activity of the active form of MMP directly or the activation/conversion of the latent form to the active form of MMP. Because MMP activity is the final result of enzyme synthesis, activation, and regulation, any observed suppressive effects of enzyme activity exerted by NSAIDs cannot yet be explained. Although the detailed inhibitory mechanism is not yet determined, these results clearly indicate that the anti-invasive effect of blocking AA metabolism through the COX pathway is associated with the inhibition of enzymatic degradative processes of tumor invasion.

Prostanoids can be viewed as local hormones that coordinate the effects of circulating hormones and other agents (e.g., collagen), which elicit prostanoid formation. In the case of PGE₂, three pharmacologically distinct prostaglandin receptors were identified (reviewed in Ref. 48), and their activation is coupled to the release of second messengers that can elicit diverse cellular activities that can alter the invasive phenotype of tumor cells. Furthermore, COX produces prostanoids, some of which have recently been shown to activate gene transcription mediated by the nuclear PPAR, a member of the steroid hormone receptor family of proteins. At least three subtypes of PPAR (α, β, and γ) have been cloned from several species, including humans, and these receptors have been implicated in tumor promotion, cellular differentiation, and apoptosis (49). PPARγ gene and protein expression are elevated in rodent colon tumors and in human colon cancer cell lines (50). In addition, Kubota et al. (51) showed that human prostate cancer cells expressed PPARγ at high levels when compared with normal prostate tissues, and that the treatment of PC-3 prostate cancer cells with a ligand for PPAR produced marked and selective necrosis of the cancer cells but not of the adjacent normal prostate cells (51). In addition, indomethacin and IB bind to, and activate, PPARγ (52), and when PPARγ is activated in macrophages, it inhibits the expression of MMP-9 (53). Therefore, it will be very interesting to understand the role of prostanoids and other AA metabolites in the regulation of gene expression, and the mechanism by which NSAIDs might affect such a regulation.

This work established an association between AA metabolism in prostate tumor cells and their proteolytic activity during invasion in vitro, a crucial step in the progression of the disease. A more detailed understanding of the mechanism by which these drugs disturb the balance between MMPs and TIMPs, as well as tests to determine whether prostate tumor cell invasion in vivo would be inhibited if animals received NSAIDs pre- and post-tumor formation, may shed more light on the potential use of NSAIDs as anti-invasive therapeutic for prostate cancer.

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