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CYCLOOXYGENASE INHIBITORS RETARD MURINE MAMMARY TUMOR PROGRESSION BY REDUCING TUMOR CELL MIGRATION, INVASIVENESS AND ANGIOGENESIS

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Tumor-derived prostaglandins (PGs) have been implicated in the progression of murine and human breast cancer. Chronic treatment with a non-selective PG inhibitor indomethacin was shown in this laboratory to retard the development and metastasis of spontaneous mammary tumors in C3H/HeJ female retired breeder mice. The present study examined the role of endogenous prostaglandins in the proliferation/survival, the migratory and invasive behavior and angiogenic ability of a highly metastatic murine mammary tumor cell line, C3L5, originally derived from a C3H/HeJ spontaneous mammary tumor. This cell line was shown to express high levels of cyclooxygenase (COX) –2 mRNA and protein as detected by Northern and Western blotting as well as immunostaining. PGE_2 production by C3L5 cells was primarily owing to COX-2, since this was blocked similarly with non-selective COX inhibitor indomethacin and selective COX-2 inhibitor NS-398, but unaffected with the selective COX-1 inhibitor valeryl salicylate. C3L5 cell proliferation/survival *in vitro* was not influenced by PGs, since their cellularity remained unaffected in the presence of PGE_2 or NS-398 or PG-receptor (EP1/EP2) antagonist AH6809; a marginal decline was noted only at high doses of indomethacin, which was not abrogated by addition of exogenous PGE_2. Migratory and invasive abilities of C3L5 cells, as quantitated with *in vitro* transwell migration/invasion assays, were inhibited with indomethacin or NS-398 or AH6809 in a dose-dependent manner; the indomethacin and NS-398-mediated inhibition was partially reversed upon addition of exogenous PGE_2. An *in vivo* angiogenesis assay that used subcutaneous implants of growth factor-reduced matrigel inclusive of tumor cells showed a significant inhibition of blood vessel formation in these implants in animals treated with indomethacin compared with animals receiving vehicle alone. These studies show that selective and nonselective COX-2 inhibitors retarded tumor progression in this COX-2-expressing murine mammary tumor model by inhibiting tumor cell migration, invasiveness and tumor-induced angiogenesis. The inhibitory effects were not entirely PG dependent; some PG-independent effects were also noted.

Key words: *cyclooxygenase; prostaglandins; NSAIDs; indomethacin; migration; invasion; angiogenesis; breast cancer*

Prostaglandins (PGs) produced by tumor cells or tumor-associated host cells (macrophages, endothelial cells and stromal cells) have long been considered to play a stimulating role in the progression and metastases of a variety of animal and human tumors including mammary tumors. Malignant breast tumors produce more PGs than normal breast tissue, and tumors with greater PG production were shown to have greater association with metastasis.[1,2] Also, patients with higher PG levels in breast tumor showed lower rates of survival after surgery.[3]

The cyclooxygenase enzymes, COX -1 and -2, synthesize PGs from arachidonic acid, an unsaturated fatty acid found in cell membrane lipids. COX-1 is constitutively expressed in most tissues leading to relatively low levels of PG production. COX-2 expression is induced in a variety of cells, *e.g.*, macrophages, after exposure to inflammatory and other stimuli (including growth factors, hormones and mitogens), leading to high levels of PG production.[4] Aberrant expression of COX-2 has been reported in murine[5] and human[6] breast cancer, cancer of the human colon,[7] lung,[8] head and neck[9] and pancreas.[10] Results from epidemiological studies suggest that use of nonsteroidal anti-inflammatory drugs (NSAIDs, *e.g.*, aspirin, indomethacin), which inhibit COX activity, reduces the risk of mortality from breast cancer[11–13] as well as colon cancer[14,15] in the human.

Spontaneous mammary tumors develop in approximately 90% of female retired breeder C3H/HeJ strain mice during their lifetime. These tumors share many features of the predominant form of human breast cancer (ductal carcinoma) in histological, invasive and metastatic properties, and COX-2 expression.[5] Chronic treatment of these mice with indomethacin delayed the development and increased the regression rate of primary tumors and reduced metastasis formation.[5] Chronic indomethacin therapy shortly after transplantation of numerous mammary tumor lines derived from C3H/HeJ spontaneous tumors had anti-tumor and anti-metastatic effects.[16] This was explained, at least in part, by a reactivation of natural killer (NK) cell activity which was suppressed by PGs in tumor-bearing mice. This suppression included inhibition of interleukin-2 (IL-2) and IFN-γ production and downregulation of their receptors on effector cells.[17] However, direct effects of the therapy on tumor growth, invasion and metastasis were not ruled out.

Our laboratory has isolated, from C3H/HeJ spontaneous mammary tumors, a number of clonally derived cell lines, varying in metastatic phenotype, and used them to examine the molecular mechanisms that underly tumor progression and metastasis. Of these, C3L5 is a highly metastatic line; both primary tumors and their metastases were found to be highly responsive to an immunotherapy protocol combining indomethacin with IL-2.[18]

The present study was designed with the C3L5 mammary tumor cell line to identify mechanisms underlying PGE_2-mediated stimulation of tumor progression. This cell line was utilized *in vitro* as well as *in vivo* to investigate whether tumor-derived PGE_2 promotes tumor cell proliferation/survival, migration (an essential component of invasion and metastasis), invasiveness and angiogenesis.

MATERIAL AND METHODS

Immunocytochemical detection of COX-2 enzyme in C3L5 cells propagated in vitro

C3L5 cells grown in complete RPMI 1640 medium (Gibco BRL, Grand Island, NY) inclusive of 10% fetal bovine serum (FBS) on chamber slides (Nunc, Naperville, IL) in a humidified chamber (37°C, 5% CO_2), were fixed in ice-cold methanol (5 min) and incubated in 3% hydrogen peroxide for 15 min to block

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endogenous peroxidase activity. Samples were incubated in 10% normal goat serum and then incubated overnight with a 1:400 dilution of polyclonal goat anti-mouse COX-2 primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). A negative control was provided by incubating 1:400 primary antibody with 10× excess control peptide (Santa Cruz). Cells were subsequently treated with biotin conjugated, donkey anti-goat IgG (Vector Laboratories, Burlingame, CA; 1:100 dilution in NBS) for 45 min at room temperature. Following this, cells were incubated in Vectastain ABC kit (Vector Laboratories). Cells were then treated with DAB chromogen for 30 sec. COX-2 expression was visualized by positive brown staining due to DAB chromogen under a light microscope.

Western blot analysis of COX enzymes

C3L5 cells were grown as above to semiconfluency. Cells were scraped off tissue culture flasks, centrifuged at 1,000*g* for 7 min and the pellet solubilized in RIPA buffer [150 mM NaCl; 50 mM Tris-HCl, pH 7.5; 1% Triton X-100; 1% deoxycholate; 0.1% sodium dodecyl sulfate (SDS); 2 mM EDTA] containing protease inhibitor cocktail tablets (complete™, Mini; Boehringer Manheim, Laval, PQ). Lysates were kept on ice for 1 hr with intermittent vortexing. Samples were then centrifuged (15,000*g*; 15 min; 4°C) to remove any insoluble material. Supernatants were used to quantify protein concentrations with BCA Protein Assay Kit (Pierce, Brockville, ON). Utilizing SDS-PAGE (polyacrylamide gel electrophoresis) (12% separating gel; 4% stacking gel) 5 μg protein/well was loaded and gel was run at 100 V for 2 hr. The proteins were then transferred to PVDF membranes (Amersham, Oakville, ON) (100 V; 1 hr; 4°C) and membranes were blocked with 5% milk in TBS-T overnight and incubated with either rabbit anti-COX -1 or -2 primary antibody (Cayman Chemical, Ann Arbor, MI; COX-1 1:500 dilution, COX-2 1:1,000 dilution, in 5% milk in TBS-T) for 1 hr. Membranes were then incubated in biotin conjugated goat anti-rabbit IgG (Vector Laboratories) (COX-1 blot-1:25,000 dilution in 5% milk in TBS-T; COX-2 blot-1:60,000 dilution, in 5% milk TBS-T) for 1 hr. This was followed by incubation in Vectastain ABC for 40 min. Chemiluminescent substrate (ECL plus; Amersham, Oakville, ON) was placed on the membranes and membranes were exposed to X-ray film (Hyperfilm ECL; Amersham, Oakville, ON).

RNA isolation and Northern blot analysis of COX-2 mRNA

RNA from C3L5 cells cultured as above was extracted by TRIZOL extraction method (Gibco BRL, Grand Island, NY). Twenty micrograms of total RNA was run on denatured agarose gel, transferred onto nitrocellulose membrane, hybridized with the EcoRI-AccI fragment of murine COX-2 cDNA (obtained from Donald Young, University of Rochester;[19]) and labeled by random priming using α^{32}P deoxy-CTP (ICN Biomedicals, Costa Mesa, CA).

PGE_2 enzyme immunoassay

Media samples from migration and invasion experiments were utilized to measure PGE_2 levels with the use of a Prostaglandin E_2 Enzyme Immunoassay Kit (Cayman Chemical). The assay was performed according to instructions supplied.

Cellularity assay

The cellularity of C3L5 cells is a measure of overall viable cell number (a composite of cell proliferation and survival) and was measured using microculture MTT assay[20] with C3L5 cells grown in media containing charcoal-stripped FBS. The different treatments included: control (10^4 cells/well + media alone); PGE_2 (ICN Pharmaceuticals) at 10–1,000 nM concentrations; indomethacin (ICN Pharmaceuticals) and NS-398 (COX-2 selective inhibitor; Biomol Research Laboratories, Inc., Plymouth Meeting, PA) at 5–100 μM; and AH6809 (PG receptor (EP1/EP2) antagonist; Biomol) at 100–10,000 nM concentrations. Also, cells were treated with indomethacin or AH6809 (at the concentrations listed above) in combination with PGE_2 at 100 nM.

Transwell migration assay

This assay was used to investigate the migratory abilities of C3L5 cells exposed to different conditions as reported earlier.[21] The transwell (Costar Corp., Toronto, Ontario) consists of upper and lower chambers that are separated by millipore membranes with 8 μm pore sizes and a surface diameter of 6.5 mm. Cells able to move to the undersurface of the millipore membrane were considered migratory. In brief, 1.5×10^4 cells plated in the upper chamber were allowed to migrate for 6–72 hr to establish the temporal kinetics of migration. Most experiments under different conditions were conducted for 24 hr when the migration was at its peak. The transwell membranes were then fixed and stained with Diff Quik® staining kit (Dade AG, Dudingen, Switzerland). Membranes were removed from transwells and cells on the undersurface of the millipore membrane were counted under a light microscope (average of 5 semi-random non-overlapping fields at 400× magnification). All treatments were performed in duplicate wells.

The different treatments (concentrations of drugs were the same both in the upper and lower chamber) in the transwell migration experiments included: control (media alone inclusive of stripped FBS); PGE_2 alone at 0.1–1,000 nM concentrations; indomethacin at 7–112 μM; NS-398 at 5–100 μM; valeryl salicylate (COX-1 selective inhibitor; Cayman Chemical) at 50 μM and AH6809 at 10–10,000 nM concentrations. Also, cells were treated with indomethacin or NS-398 or AH6809 (at the concentrations listed above) in combination with exogenous PGE_2 at 100 nM.

Transwell invasion assay

The invasion assays were carried out for 24–72 h, and were identical to the migration assays except for the inclusion of a Matrigel™ barrier, which the cells had to degrade prior to migration.[21] The millipore membranes were coated with 120 μl of a 1 in 60 dilution of growth factor-reduced Matrigel™ (Collaborative Biomedical Products, Bedford, MA) in PBS (~0.12 mg/ml) and the transwell plates were incubated overnight (37°C; 5% CO_2) in a closed environment to allow even gelification of the diluted Matrigel™ prior to the assay.

The treatments in the invasion assays (indomethacin and AH6809) were identical to those in the migration assays. However, 2.5×10^4 (instead of 1.5×10^4) C3L5 cells were added per well. A 48 hr time point was chosen for most treatment conditions on the basis of pilot experiments showing peak invasion index at this time point.

Gelatin zymography

Conditioned media from C3L5 cells cultured for 24 hr in media containing stripped serum was used in running the protein gel. Ten microliters of media was combined with 20 μl of 2× sample buffer and mixed. The samples were then loaded into precast 10% gelatin Ready Gels (Bio-Rad Laboratories, Mississauga, Ontario). Gels were run at 100 V for 2 hr. Gels were incubated in 100 ml of Renaturation Buffer (2.5% Triton X-100 in deionized H_2O) with agitation. Next, the gels were incubated at 37°C in 100 ml of Bio-Rad Zymogram Development Buffer (Bio-Rad Laboratories) overnight.

Gels were then stained with 0.5% Coomassie Blue R-250 (Bio-Rad Laboratories) in 40% methanol/10% acetic acid for 1 hr at room temperature. Destaining followed for 4 hr with occasional changing of destaining solution (40% methanol/10% acetic acid in deionized water). Clear bands in the gel, indicating proteolytic activity, were visualized by computer imaging.

Tumor-induced in vivo *angiogenesis assay*

Female C3H/HeJ mice (8–10 weeks of age; Jackson Laboratories, Bar Harbour, ME) were used as hosts for *in vivo* angiogenesis experiments. The mice were housed in animal quarters at the University of Western Ontario (London, Ontario) under a 12 hr light/dark cycle according to Canadian Council on Animal Care Guidelines. Standard rodent chow and tap water were provided unless otherwise indicated.

The level of angiogenesis *in vivo* was assessed with a novel *in vivo* assay devised in our laboratory.[22] In brief, C3L5 tumor cells (5×10^4) suspended in growth factor-reduced Matrigel™ (~4 mg/0.5 ml RPMI) were injected subcutaneously in the left inguinal region (day 0). Matrigel™ injections containing no cells were used as negative controls in the right inguinal area. The implants solidified at body temperature. Implant bearing mice (n=10 per group) were treated chronically with either COX inhibitor indomethacin (12 μg/ml in drinking water (0.2% ethanol) or drinking water containing vehicle (0.2% ethanol) alone (from day −1). Treatments continued until day 14 and drinking water intake was measured for the duration of the experiment to ascertain that the intake was identical in indomethacin and vehicle consuming mice. At the end of 14 days, implant growth was measured by taking readings of implants at their greatest and smallest diameters. Mice were then sacrificed and implants recovered, fixed in 4% paraformaldehyde overnight and paraffin-embedded. Implant cross-sections were stained with Masson's trichrome and coded. Images of implant sections were captured with the Northern Eclipse computer imaging program (Empix Imaging (Mississauga, Ontario) at 160× magnification and blood vessels were visualized and counted utilizing Mocha image analysis software (Jandel, San Rafael, CA). The mean of the 3 "hottest" (most abundant) blood vessel fields for each implant was used as the measure of angiogenesis.

Statistical analysis

Data were analyzed using the Sigma Stat 2.0 (Jandel) statistics program. Data comparing 2 means were tested by Student's *t*-test; those comparing multiple means for a single effect were tested using 1-way ANOVA and those comparing multiple means for dual treatment experiments were tested using 2-way ANOVA. A probability of 0.05 was considered statistically significant.

RESULTS

COX protein and mRNA expression by C3L5 cells

Figure 1*a* depicts the immunoreactive COX-1 and -2 protein bands (both at ~70 kDa region) obtained with C3L5 cells under normal culture conditions. COX-2 bands are more intense in comparison to COX-1 bands. C3L5 cells appear to have a high abundance of COX-2 protein. Figure 1*b* depicts the Northern blots for detection of COX-2 mRNA. A strong expression of COX-2 gene in C3L5 cells is apparent both *in vitro* and *in vivo*. Figure 1*c* depicts the presence of immunoreactive COX-2 protein in C3L5 cells cultured in normal growth media. Antibody treatment resulted in intense staining in the peri-nuclear region and nuclear membrane with diffuse staining throughout the remaining cytoplasm. No staining is apparent in the negative control (treated with antibody pre-absorbed with excess control peptide).

Roles of COX-1 vs. *COX-2 on PGE_2 production*

To determine the effects of the different COX inhibitors on the production of PGE_2 by C3L5 cells, media conditioned from treated C3L5 cells in the 24 hr migration assays were used in a PGE_2 enzyme immunoassay. Treatments included: control, non-selective COX inhibitor indomethacin and selective COX-2 inhibitor NS-398 at 5–50 μM concentrations, and selective COX-1 inhibitor valeryl salicylate at 10–500 μM concentrations. Table I summarizes these results. Valeryl salicylate-treated cells had nearly similar PGE_2 concentrations in the media as control treatments even at the highest drug concentration (500 μM). Indomethacin and NS-398 treated cells exhibited significantly lower PGE_2 concentrations in the media. Maximal inhibition was achieved with indomethacin at 10 μM (IC_{34}) and NS-398 at 5 μM (IC_{46}). It is likely that cells made significant levels of PGE_2 shortly after plating, prior to and during the entry of these drugs to exert their activity within the cells. This possibility was validated with a subsequent experiment (data not presented) in which the supernatant of cells subjected to various treatment conditions as in Table I were removed at 3 hr after incubation and cells were treated with the same agents in fresh medium for a period of 24 hr. Both experiments revealed that PGE_2 production by C3L5 cells was essentially a consequence of COX-2 expression.

Role of endogenous/exogenous PGE_2 on C3L5 cell proliferation/survival, migration and invasion in vitro.

These functions were tested with cellularity, migration and invasion assays conducted *in vitro*. In initial experiments, the temporal kinetics of migration (6–72 hr) were established for C3L5 cells. The migration index peaked at 24 hr followed by a small decline at 48 hr and then further decline at 72 hr (data not presented). Consequently, a 24 hr incubation period was chosen for presenting data on all cellularity and migration assays carried out under different treatment conditions. This allowed us to test or exclude the possibility that the treatment effects on migration were a consequence of the effects on cellularity. Invasion indices, however, peaked at 48 hr, most likely because the cells need extra time to degrade and transgress the Matrigel™ barrier. This time point is presented for invasion under various treatment conditions.

*Cellularity.*The results on cellularity at 24 hr under different treatment conditions are shown in Table II. Cellularity measures in these experiments reflected the combined effects on cell proliferation and survival. None of the treatments presented in this table affected cell viability (trypan blue exclusion) over a 6 hr incubation period.

Exogenous PGE_2 at a wide range of concentrations (10–1,000 nM) had no effect on the cellularity of C3L5 cells. Indomethacin did not affect C3L5 cellularity at low (5–10 μM) concentrations. However, minor but significant decline in cellularity was evident at higher concentrations (50–100 μM). This decline was not abrogated with the addition of exogenous PGE_2, indicating that the effects were non-PGE_2 related and may be due to toxicity, although not identifiable in viability assays. Treatment with NS-398 (5–100 μM) had no significant influence on C3L5 cellularity. Additional presence of PGE_2 (100 nM) made no difference in the results.

Finally, C3L5 cellularity was unaffected in the presence of varying concentrations of AH 6809, a PGE_2-receptor (EP1/EP2) antagonist. This was not due to a lack of biological activity of this inhibitor, since in later experiments (migration and invasion assays) an effect was noted. Additional presence of PGE_2 (100 nM) did not influence the results (Table II). Valeryl salicylate, the selective COX-1 inhibitor, which proved to be ineffective in blocking PGE_2 production by C3L5 cells (Table I) was found to be moderately toxic (80% viability) at a concentration of 100 μM and highly toxic to these cells at higher concentrations, so that most of the cells died by 24 hr (data not shown).

Above results taken together suggest that exogenous or endogenous PGE_2 did not have a significant effect on C3L5 cell proliferation/survival.

*Migration.*The results of transwell migration assays in the presence of various agents for 24 hr are shown in Figure 2. The cells used in the migration experiments presented here are from the same cohort as those used in the respective cellularity assays previously presented (Table II). The data represent one of 3 experiments with essentially similar results.

Varying concentrations of exogenous PGE_2 alone (0–1,000 nM) had no influence on C3L5 cell migration (Fig 2*a*). Indomethacin, at concentrations of 14–112 μM significantly reduced migration of C3L5 cells. The addition of PGE_2 (100 nM) to indomethacin treated cells was found to significantly (but incompletely) abrogate the inhibitory effects of indomethacin at higher (28–112 μM) but not lower (14 μM) inhibitor concentrations in this particular experiment. In other experiments, a partial abrogation was also noted at 14 μM concentration of indomethacin. In Figure 2*c* NS-398 (5–100 μM), a COX-2 selective inhibitor, was found to dose-dependently reduce mi-

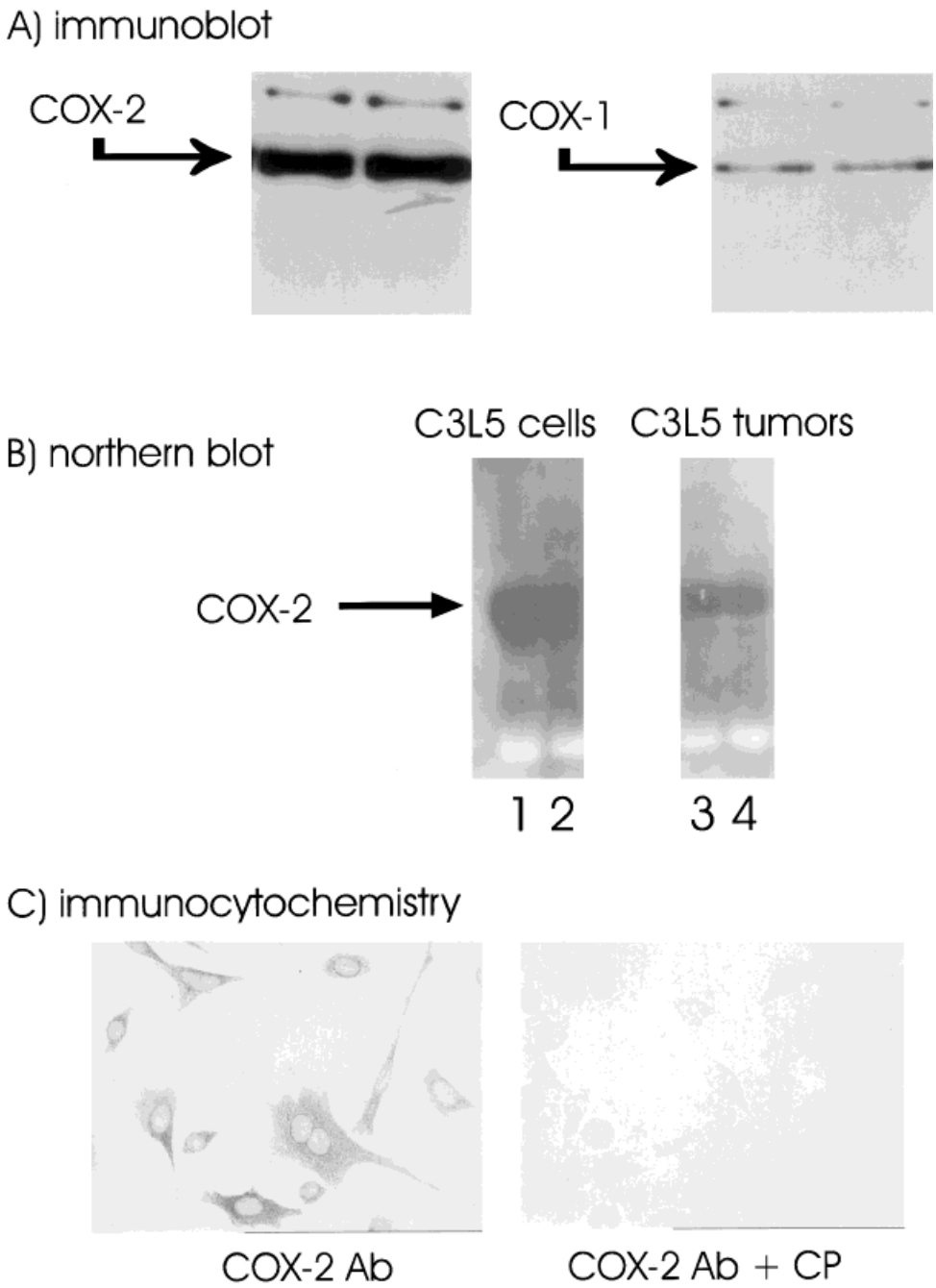

FIGURE 1 – Expression of COX in C3L5 cells cultured *in vitro*. (*a*) Representative Western blots detecting COX-2 and COX-1. (*b*) COX-2 mRNA expression in C3L5 cells and tumors. A single transcript of ~4.2 kb was identified in these Northern blots. Lanes 1 and 2 indicate different samples of RNA obtained from C3L5 cells cultured under normal conditions. Lanes 3 and 4 indicate different samples of RNA isolated from C3L5 tumor transplants grown in C3H/HeJ mice. An abundance of COX-2 mRNA in C3L5 cells is apparent in both cases; a lower abundance noted in the transplant-derived C3L5 tumors may be attributed to the presence of other host-derived cells in these tumors. (*c*) Immunochemical localization of COX-2 expression in C3L5 cells. Cells were treated with 1:400 dilution of polyclonal COX-2 antibody followed by 1:100 dilution of secondary (donkey anti-goat) antibody. Intense staining is visible in the perinuclear region and at the nuclear envelope with diffuse staining in the remainder of the cytoplasm. For negative control, primary antibody was pre-absorbed with excess control peptide (CP).

gration levels of C3L5 cells. Additional presence of exogenous PGE_2 (100 nM) completely abrogated the migration inhibiting effects of NS-398 at lower (5–20 μM) inhibitor concentrations. The abrogation was incomplete at higher inhibitor concentrations (50 and 100 μM).

Figure 2*d* shows that the PG-receptor antagonist, AH6809, significantly inhibited C3L5 cell migration at all concentrations (10–10,000 nM) in a concentration-dependent manner. As expected, an additional presence of exogenous PGE_2 (100 nM) was unable to abrogate the drug-induced inhibition of C3L5 cell migration. These results indicated migration promoting effects of endogenous PGE_2 acting via EP1/EP2 receptors.

On only one occasion (Fig. 2*d*, but not in Fig. 2*a–c*, or in 2 other experiments not presented here), PGE_2 alone (100 nM) caused a marginal decline in migration, indicating that this was not a reproducible phenomenon.

TABLE I – PGE_2 MEASUREMENTS OF 24 HR MIGRATION ASSAY SUPERNATANTS OF C3L5 CELLS SUBJECTED TO DIFFERENT TREATMENTS

PGE_2 concentration index (% of control)[1]					
Valeryl salicylate		Indomethacin		NS-398	
10 μM	105 +/− 15	5 μM	83 +/− 7	5 μM	54 +/− 6
100 μM	86 +/− 3	10 μM	66 +/− 5	10 μM	68 +/− 8
500 μM	103 +/− 16	50 μM	64 +/− 4	50 μM	63 +/− 4

[1] PGE_2 concentration accumulated at 24 hr was 554 pg/ml in control cells subjected to no treatment.

TABLE II – CELLULARITY OVER 24 HR OF C3L5 CELLS EXPOSED TO VARIOUS TREATMENT CONDITIONS

	Cellularity index (% of control)	
Treatment[1]		+100 nM PGE_2
Control	100	
PGE_2		
10 nM	95 +/− 2	—
100 nM	97 +/− 2	—
1000 nM	108 +/− 1	—
Indomethacin		
5 μM	110 +/− 2	104 +/− 3
10 μM	105 +/− 2	98 +/− 4
50 μM	83 +/− 3[2]	80 +/− 2
100 μM	77 +/− 4[2]	76 +/− 4
NS-398		
5 μM	95 +/− 5	106 +/− 3
10 μM	99 +/− 4	104 +/− 3
50 μM	94 +/− 10	101 +/− 3
100 μM	94 +/− 2	93 +/− 2
AH6809		
100 nM	100 +/− 3	97 +/− 4
1000 nM	103 +/− 4	99 +/− 14
10000 nM	85 +/− 7	95 +/− 9

[1] Following the treatments, MTT and extraction buffer reagents were added followed by further incubation. Plates were read the following day in a plate reader at 570 nm. n = 4 per treatment. Cellularity is expressed as an index (% of control values). Data reflect mean +/− standard error (SE).–[2] Significantly ($p < 0.05$) lower than control.

The fact that the effects on C3L5 cellularity (same cohort of cells) by various treatments were not in parallel to those noted on C3L5 cell migration (Table II *vs.* Fig. 2) provides a convincing argument that the effects on migration were not a consequence of the effects of these drugs on cellularity.

Invasion. Invasion indices of C3L5 cells at 48 hr under different treatment conditions are presented in Figure 3. The data represent 1 of 2 experiments with essentially similar results.

Figure 3*a* shows that indomethacin concentrations of 14–112 μM significantly inhibited C3L5 cell invasiveness in a concentration dependent manner and this inhibition was partially abrogated with exogenous PGE_2 (100 nM) only at higher indomethacin concentrations (56 and 112 μM). These results suggested that endogenous PGE_2 stimulated C3L5 invasiveness; however, invasion inhibiting effects of indomethacin were only partially ascribable to PGE_2 inhibition.

Figure 3*b* shows the effects of varying concentrations of PG-receptor antagonist (AH6809) on C3L5 cell invasion. This drug significantly inhibited invasiveness only at the 10,000 nM concentration. As expected, the inhibition was not reversed in the presence of exogenous PGE_2. Thus invasion stimulation of endogenous PGs appears to be mediated by EP1/EP2 receptor(s).

Comparison of the effects of multiple drugs

Since there was the possibility of some variability from one experiment to another in terms of cell cycle status of C3L5 cells used, which might influence their migratory and invasive behavior, a set of experiments was performed in which drug treatments could be compared against one another using the same cell populations and equivalent drug concentrations. In addition, valeryl salicylate, a COX-1 selective inhibitor, was included to determine whether the COX-1 isoform played a role in the cellular processes studied. The concentration of 50 μM was chosen for the COX inhibitors on the basis of our observation that at higher concentrations, valeryl salicylate was toxic to C3L5 cells. PG receptor antagonist AH6809 was used at 10,000 nM concentration.

Cellularity. Cellularity assays revealed no significant effect of indomethacin, valeryl salicylate, NS-398 or AH6809 at the above specified concentrations on C3L5 cellularity (data not presented).

Migration. Figure 4*a* presents the results of a 24 hr migration assay performed with multiple drugs. All treatments except for valeryl salicylate significantly inhibited C3L5 migration in this assay.

Invasion. Figure 4*b* depicts the results of a multiple treatment invasion assay at 48 hr. Drug concentrations were identical to that in the migration assay. All treatments except with valeryl salicylate significantly inhibited C3L5 invasion in this assay.

These experiments reinforced the conclusion from earlier experiments that endogenous PGE_2 promoted C3L5 cell migration and invasiveness by utilizing EP1/EP2 receptors. In addition, the endogenous PGE_2-mediated effects appeared to be primarily due to COX-2 activity.

Effect of PGs on MMP activity

Figure 5 shows the zymography gel containing samples of media from C3L5 migration assays. The clear bands indicate the presence of MMP-2 (thicker band; 72 kDa) and MMP-9 (thinner band; 92 kDa). There was no visible difference in band appearance from one treatment to another. These results may indicate that PGs did not regulate MMP activity in C3L5 cells or that the zymography was not sensitive enough to detect small differences in MMP activity due to the treatments.

Effects of indomethacin therapy on C3L5 induced angiogenesis in vivo

C3L5 cells suspended in growth factor-reduced Matrigel™ were implanted subcutaneously into C3H/HeJ mice (which received indomethacin or vehicle alone in the drinking water for the whole experimental duration) and implants were harvested at 14 days. Figure 6*a* shows that indomethacin treatment of the host over a 14-day period significantly inhibited the growth of implants, as noted from the relative implant size (given by the product of maximal and minimal diameters).

Figure 6*b* summarizes the data on measurements of angiogenesis for 3 different treatment groups; control (C3L5 cells in Matrigel™; animals drinking vehicle alone), indomethacin treated (C3L5 cells in Matrigel™; 12mg/ml indomethacin in drinking water) or Matrigel™ alone (no cells) (combining implants in control and indomethacin-treated mice).

Very few vessels were detectable in Matrigel™ only implants within vehicle or indomethacin treated mice confirming earlier work from this laboratory that growth factor-reduced Matrigel™ had no significant angiogenic ability on its own.[22] Tumor cell inclusive implants in control mice exhibited high levels of tumor-induced angiogenesis. This was significantly inhibited in indomethacin-treated mice indicating an angiogenesis-stimulating role of C3L5 cell derived PGs.

DISCUSSION

Our study was conducted with the highly metastatic C3L5 mammary adenocarcinoma cell line derived in this laboratory from a spontaneous C3H/HeJ mouse mammary tumor, sharing many similarities with the aggressive forms of human breast cancer.[5] It was utilized *in vitro* as well as *in vivo* to investigate the role of endogenous PGs in tumor progression by measuring specific cel-

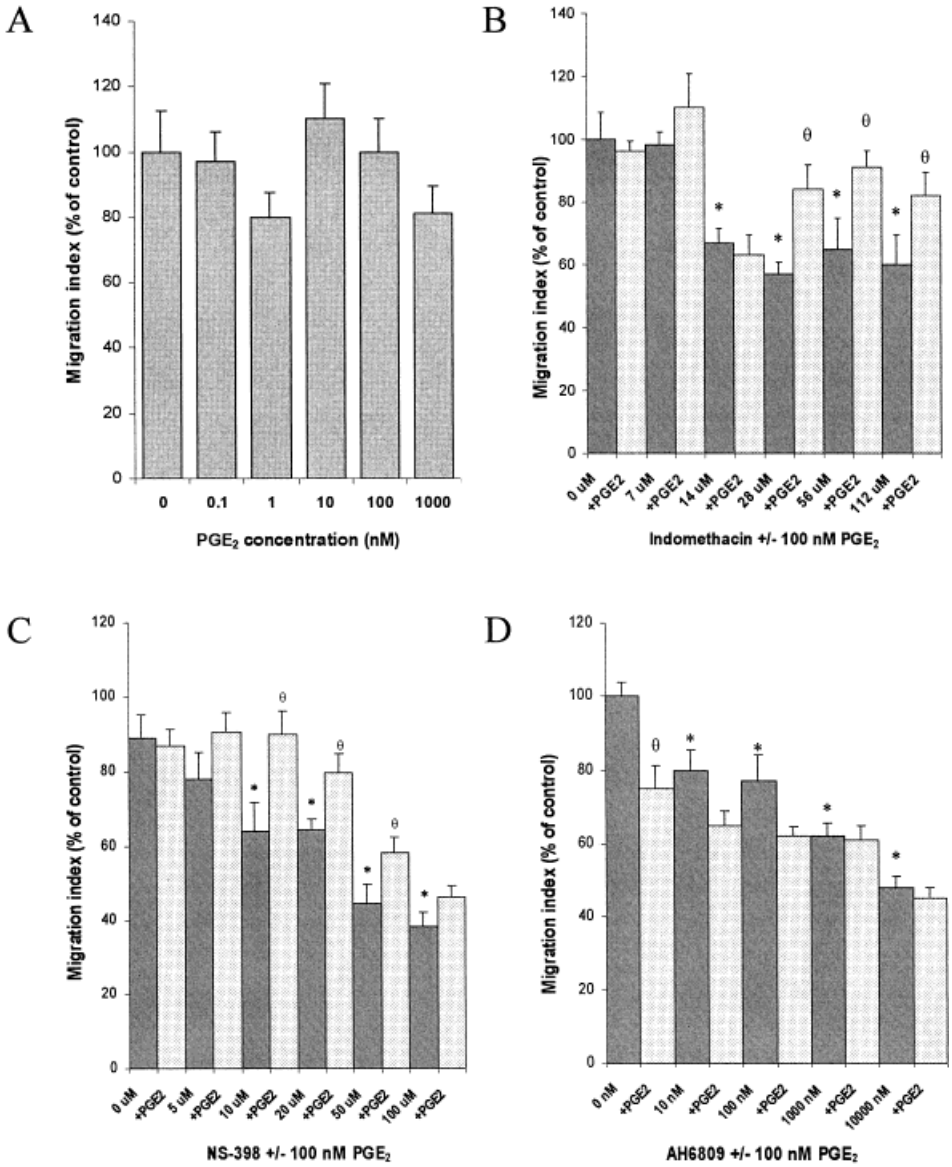

FIGURE 2 – Migration assays. C3L5 cells were treated with various concentrations of PGE_2 (0–1,000 nM), indomethacin (0–112 μM) ± PGE_2 (100 nM) or NS-398 (0–100 μM) ± PGE2 (100 nM) or AH6809 (0–10,000 nM) ± PGE_2 (100 nM) during the 24 hr migration assay. Values represent the mean cell counts (± SE); n=10. (*a*) With PGE_2 treatment, there was no significant difference between any of the treatments inclusive of the controls (tested through one way ANOVA). (*b*) Indomethacin significantly suppressed migration at 14–112 μM concentrations (*). Additional presence of PGE_2 (100 nM) significantly abrogates this suppression, partially restoring migration levels in comparison with indomethacin treatment alone (θ) (tested by 2-way ANOVA). (*c*) NS-398 significantly suppressed migration at 10–100 μM concentrations (*) in a concentration dependent manner. Addition of exogenous PGE_2 (100 nM) significantly abrogated the inhibitory effect of NS-398 at 10–50 μM concentrations (θ) (tested by 2-way ANOVA). (*d*) There was a concentration dependent decline (*) in migration index resulting from AH6809 treatment, which was not affected by the addition of PGE_2. In this experiment alone, there was a minor but significant (θ) drop in migration in the presence of PGE_2 alone as compared with untreated cells (tested by 2-way ANOVA).

lular processes including proliferation/survival, migration, invasion and tumor-induced angiogenesis.

The bulk of the earlier evidence for the stimulatory role of tumor-derived PGs in tumor progression have been primarily correlative, *e.g.*, a positive association with tumor grades or metastasis in human breast[1,2,23] and colon[24] cancers, and negative association with patient survival after breast cancer surgery.[3] More recently, the expression of COX-2, the enzyme primarily responsible for elevated PG production, has been found to be upregulated in breast,[6] colon[7,25,26] and other cancers.[8–10] A large body of evidence has shown PGs to have immune suppressive roles in cancer indicating host-related mechanisms responsible for tumor progression.[15,17,25,28] However, studies that investigated the direct role of PGs in tumor-related mechanisms, *i.e.*, tumor cell functions that are required for tumor progression, have been limited.[29–31] The present study in our murine mammary tumor model revealed that tumor-derived PGs owing to COX-2 expression by tumor cells stimulated their migration and invasiveness as well as tumor-induced angiogenesis. Because addition of exogenous PGE_2 did not always fully restore the decreased cell functions, such as migration and invasiveness caused by the COX inhibitors, it is apparent that these inhibitors may also exert PG-independent effects. While we have largely excluded toxicity on the basis of

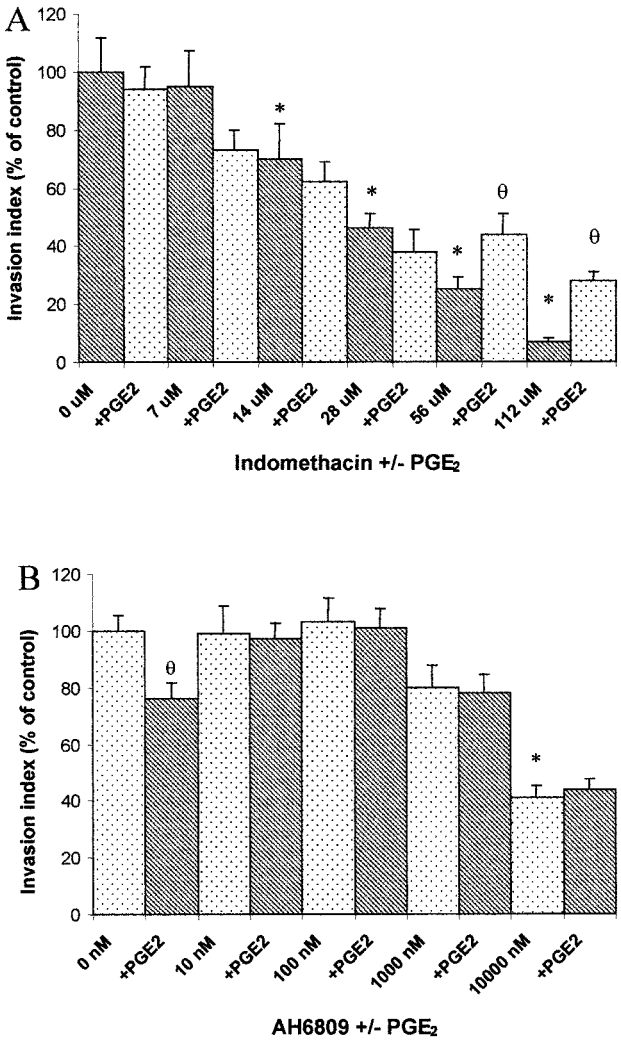

FIGURE 3 – Invasion assays. Forty-eight hour invasion assays were conducted with C3L5 cells in the presence of varying concentrations of indomethacin (0–112 μM) or AH6809 (0–10,000 nM) in the presence or absence of PGE_2 (100 nM). Data represent mean number of cells (± SE); n=10. (*a*) Significant inhibition (*) as compared with control was noted with indomethacin treatment at 14–112 μM concentrations. Significant abrogation of this inhibition (θ) due to additional presence of PGE_2 was only noted at higher indomethacin concentrations (tested through 2-way ANOVA). (*b*) Significant decline in invasiveness (*) as compared with the control was noted with AH6809 treatment only at the highest concentration. Additional presence of PGE_2 (100 nM) did not influence the results. Presence of PGE_2 alone in this experiment resulted in a minor but significant (θ) drop in invasiveness (tested through 2-way ANOVA).

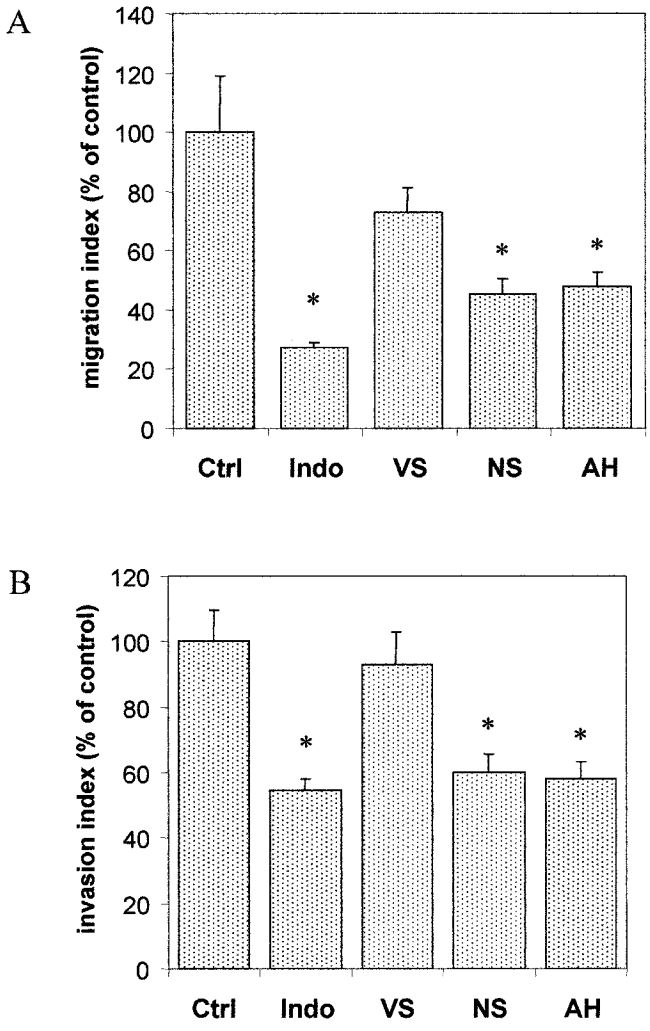

FIGURE 4 – Migration and invasion assays with multiple drug treatments. Migration assays (24 hr) and invasion assays (48 hr) were conducted in media alone (controls) or in the presence of indomethacin (Indo) (non-selective inhibitor), valeryl salicylate (VS) (COX-1 selective inhibitor) and NS-398 (NS) (COX-2 selective inhibitor), all at a concentration of 50 μM; and AH 6809 (AH) (10,000 nM). Data were normalized to 100 % for control values and represent means ± SE; n=14. Significant differences from control values are indicated by (*) (tested by 1-way ANOVA). All treatments, except for valeryl salicylate caused a significant decrease in migration index (*a*) and invasion index (*b*).

trypan blue exclusion and cellularity assays, the nature of the PG-unrelated effects remains undetermined at present.

Cyclooxygenase expression

Present findings of abundant COX-2 mRNA and protein expression by C3L5 cells *in vitro* and *in vivo* are consistent with the in situ findings of the presence of immunoreactive COX-2 protein in the primary C3H/HeJ spontaneous mammary tumors.[5] PGE_2 production by C3L5 cells in the presence of selective and non-selective COX inhibitors established that it was primarily owing to COX-2. Immunocytochemical localization of COX-2 protein in C3L5 cells was found to be high in the peri-nuclear and nuclear envelope regions. Expression of COX-2 protein in NIH/3T3 cells has been shown to occur in a similar fashion.[32] The 18 amino acid carboxy terminus sequence found in COX-2, but not in COX-1, may be required to target COX-2 or its insertion in the nuclear envelope.[32] The presence of COX-2 protein at the nuclear envelope region may indicate a role for PGs produced by the enzyme in nuclear activities including the regulation of cell proliferation and differentiation.[33]

Prostaglandins and cell proliferation/survival

An absence of effects of various concentrations of exogenous PGE2 on C3L5 cellularity, *i.e.*, cell proliferation and/or survival, may be due to a masking of the effects in the presence of very high levels of endogenous PGE_2 produced by these cells, owing to high COX-2 expression as shown in Table I. Studies in other cell systems have provided conflicting relationships between PGs and cell proliferation, including stimulatory,[34,35] inhibitory[35,36] or indifferent.[37] Furthermore, COX-2 and not COX-1 activity has been attributed to stimulation of proliferation of normal and tumorigenic

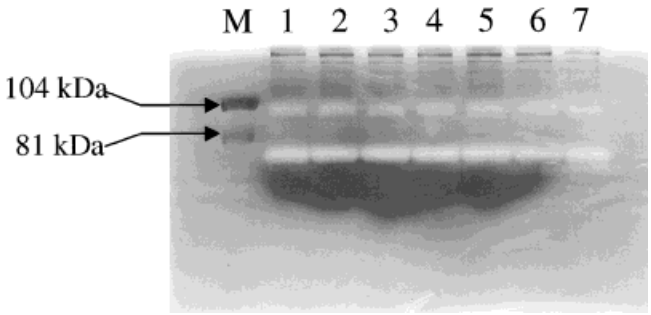

FIGURE 5 – Gelatin zymography for detection of matrix metalloproteinases. Zymography gel containing samples of media from C3L5 migration assays. Lane M indicates molecular weight markers. Numbered lanes describe C3L5 conditioned media samples of cells exposed to different drug treatments (all 50 μM, except for AH6809-10,000 nM): 1=control, 2=indomethacin, 3=valeryl salicylate, 4=AH6809, 5=NS-398, 6=indomethacin + 100 nM PGE_2, and 7=NS-398 + 100 nM PGE_2. The clear bands indicate the presence of MMP-2 (thicker band; 72 kDa) and MMP-9 (thinner band; 92 kDa). There was no visible difference in band appearance from one treatment to another.

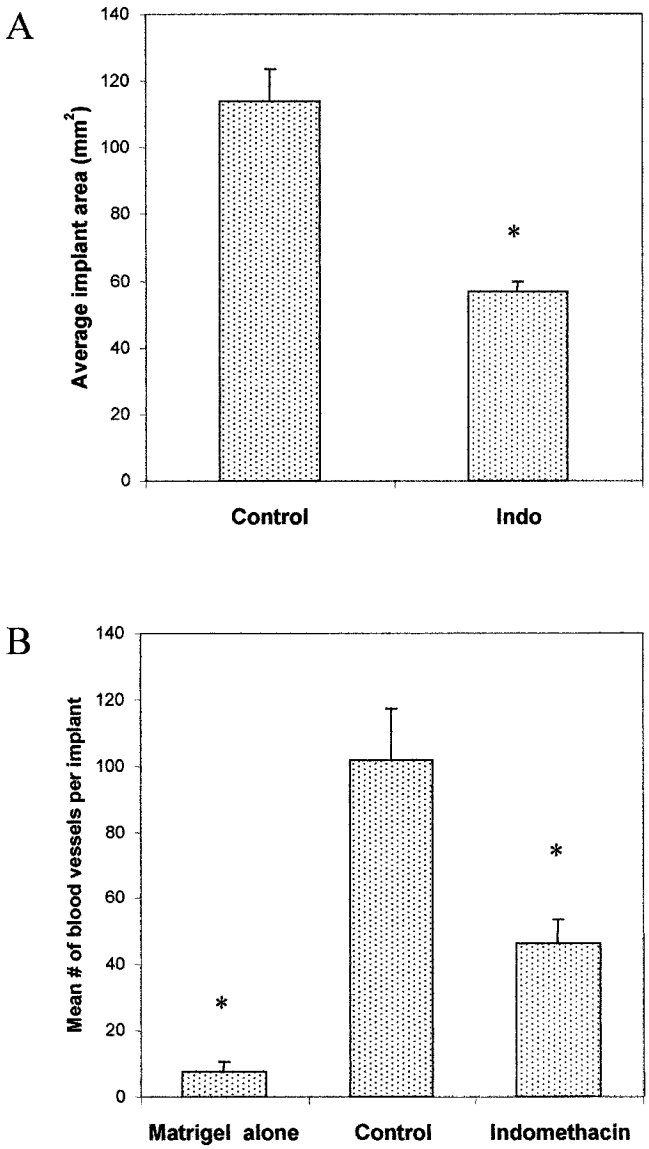

FIGURE 6 – *In vivo* angiogenesis assay. (*a*) The relative mid cross-sectional areas of implants were measured by multiplying the minimum and maximum diameters of implants. Diameter measurements were performed with calipers. (*) indicates a statistically significant difference (measured by *t*-test). (*b*) The level of angiogenesis of implants (C3L5 cells suspended in growth-factor reduced Matrigel™) in mice treated with vehicle (control-0.2% ethanol in water) and mice subjected to indomethacin treatment [12 mg/ml in water (0.2% ethanol)] was tabulated. The cross-sectional area of the tumor was non-selectively divided into several microscope fields at 160× magnification. The number of blood vessels visible in each field were then counted with the aid of the Mocha Imaging system. The average of the 3 "hottest" fields per implant was used as a measure of their level of angiogenesis. Depicted on the graph are the means (± SE) of these "hot spots" for each group of animals. n=10 for each group (*) indicates a significant difference from control (measured by 1-way ANOVA).

intestinal epithelial cells *in vitro*.[38] The presence or absence of functional PG receptors[39] may explain some of the differences in results.

In the present study, a small decline in C3L5 cellularity in the presence of high concentrations of non-selective COX inhibitor indomethacin appeared to be unrelated to COX inhibition, since exogenous PGE_2 failed to restore cellularity to control levels. The findings that PG-receptor antagonist AH6809, which affected cell migration and invasiveness but not cellularity, lent further support to the contention that PGs had no significant influence on C3L5 tumor cell proliferation/survival *in vitro*.

Prostaglandins and cell migration

Cellular migration is an essential step for invasion and metastasis. Exogenous PGE_2 alone at different concentrations had no significant effect on C3L5 cell migration. This finding may likely be attributed to the masking effects of high levels of endogenous PGs released by C3L5 cells in the medium. This possibility was validated by the findings that the non-selective COX inhibitor indomethacin (56–112 μM) led to significant reduction in migration, which was partially abrogated by addition of exogenous PGE_2. That migration stimulating effects of endogenous PGE_2 were primarily a result of COX-2 activity was evidenced by the migration inhibition with the selective COX-2 inhibitor NS-398 and an abrogation of this inhibition with exogenous PGE2 at lower inhibitor concentrations. The findings that treatment with the PG-receptor antagonist, AH6809, also inhibited C3L5 cell migration suggested that the migration promoting effects of endogenous PGs were mediated by EP1/EP2 receptors. These results are consistent with the report by Young *et al.*[29] that show that a Lewis Lung Carcinoma cell line responded by migration stimulation in the presence of exogenous PGE_2 as well as PGE_2 produced by another Lewis Lung Carcinoma cell line.

The mechanisms of migration stimulation in cancer cells by endogenous or exogenous PGE_2 remain to be investigated. In the present study, it was evident that EP1/EP2 receptors were involved. Post-receptor signaling pathways may involve cAMP as the second messenger or increased Ca^{++} mobilization, as has been established for other PGE_2 actions.[40]

Prostaglandins and cellular invasiveness

Treatment with indomethacin, or the PG-receptor antagonist AH6809, resulted in inhibition of C3L5 cell invasion at higher drug concentration levels. Selective COX-2 inhibitor NS-398 also had anti-invasive effects. Exogenous PGs were able to partially abrogate the effects of indomethacin, but not of AH6809. These results reveal that endogenous PGs promote C3L5 cell invasiveness and that this was primarily due to COX-2 activity.

The present results of the migration and invasion experiments demonstrated similar patterns in terms of drug and PG effects. Thus it is possible that the effects observed in the invasion studies are largely attributable to migration-related effects, rather than matrix degradation by tumor cells, since invasion requires both

cellular processes. This contention is consistent with the lack of effects of COX inhibitors and the PG receptor antagonist on cellular MMP activity. However, zymography may not be sensitive enough to suggest whether endogenous PGE_2-modulated MMP expression by C3L5 cells. Further studies are needed in these cells to test whether PGs have an effect on the transcription of MMP or TIMP (natural inhibitors of MMPs) genes.

A number of studies have attempted to clarify the role of PGs in cancer cell invasiveness. Linoleic acid, one of the precursors of AA, stimulated human breast cancer cell invasion *in vitro* and this stimulation was suppressed by indomethacin.[30] Linoleic acid addition also stimulated the expression of type IV collagenase, one of the key extracellular matrix degradative enzymes, in human breast cancer cells.[41] The treatment of prostate tumor cells with the COX-2 selective inhibitor NS-398 inhibited the secretion of pro-MMP-2, MMP-2 and pro-MMP-9 by these cells.[42] COX-1 or COX-2 overexpression in a human breast cancer cell line stimulated induced expression of MMP-2 and MT-1 MMP.[43] Finally, tumor-derived PG can also have a paracrine effect in stimulating the production of hepatocyte growth factor by stromal cells which may in turn stimulate uPA activity in tumor cells.[44]

Prostaglandins and tumor-induced angiogenesis

PGs have long been known to induce angiogenic responses.[45] Results herein. using indomethacin therapy, established that endogenous PGs promoted tumor-induced angiogenesis. However, we have not established the relative contributions of COX-2 *vs.* COX-1 in the angiogenesis response. In other studies, mostly COX-2 and to a minor extent COX-1 have been implicated in angiogenesis. For example, the COX-2 selective inhibitor NS-398 inhibited colon cancer cell-induced migration of endothelial cells,[31] the cells responsible for the development of new blood vessels. Treatment of these endothelial cells with COX-1 antisense oligonucleotides inhibited COX activity and also endothelial cell tube formation, an addition[3]1 that indicates that COX-1 may play a complimentary role. In contrast, angiogenesis in a corneal angiogenesis model was inhibited with COX-2 selective inhibitors celecoxib[46] and NS-398[47] but not with a COX-1 selective inhibitor.[46]

As solid tumors grow, areas in the interior of the tumor furthest from the blood supply are exposed to hypoxic conditions due to lack of adequate circulation. Hypoxia is known to induce the expression of COX-2 mRNA and protein in human vascular endothelial cells.[48] The COX-2 gene has a hypoxia-responsive element and this fact may be of importance in tumor biology. Present study along with others[31] have shown the importance of PG induction in stimulating angiogenesis. Another angiogenic gene, VEGF, also has a hypoxia-responsive element,[49] which can mediate transcriptional regulation of Nitric Oxide.[50] Nitric Oxide is a potent angiogenic molecule also involved in the progression of many tumors, including the presently studied C3L5 mammary tumor.[22] Because the interior cells in a tumor are exposed to hypoxic conditions, induction of COX-2 as well as VEGF may stimulate angiogenic responses.

Prostaglandins vs. *Nitric Oxide (NO) in tumor progression*

Tumor-derived NO, resulting from expression of constitutive or inducible NO synthase enzymes in tumor cells or tumor-associated cells, has also been shown to promote the tumor progression[51] in the case of a variety of human[52] and animal tumors including the C3L5 mammary tumor cells, which express endothelial type (e) NOS.[53] The mechanisms of NO action in C3L5 cells were found to be identical to that of PGs such as stimulation of migration, invasiveness and angiogenesis.[21,22] Since NO and PG can have reciprocal modulating effects in a variety of cells,[54] it will be important to determine the nature of interaction between the 2 molecular species in stimulating tumor progression in the present model.

COX inhibitors in cancer therapy, chemoprevention and chemointervention

Owing to the importance of COX-2 in colon and numerous other cancers, there has been a recent interest in the use of selective COX-2 inhibitors, because of their relative freedom from toxicity, for inclusion in therapeutic regimens. Moore *et al*.[55] reported that COX-2 selective inhibitor, celecoxib, in combination with 5-fluorouracil or cyclophosphamide greatly enhanced the anti-tumor effects of chemotherapy in a colon cancer model. In another tumor model, COX-2 selective inhibitors have also shown promise in combination with radiation therapy, enhancing tumor radiation responses.[56] Finally, celecoxib has recently been shown to have chemopreventive effects against the development of chemically induced mammary tumors in the rat.[11] These studies, combined with the present study and the report of aberrant COX-2 expression in human breast cancer,[6] suggest that selective COX-2 inhibitors will have an important role in chemoprevention, chemointervention and therapy of human breast cancer. Further studies are also needed to define the PG-independent effects of some of the COX inhibitors.

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