Abstract. Cyclooxygenase-2 (COX-2) increases breast cancer cell invasion. Expression of various pro-angiogenic and pro-invasive factors has been correlated with high expression of COX-2. However, whether these factors are essential to COX-2-mediated breast cancer invasion, and the mechanisms by which COX-2 increases the expression of these factors are unknown. Our microarray results indicate that higher COX-2 expression was associated with increased levels of interleukin-8 (IL-8), a key factor in breast cancer invasion and metastasis. COX-2 overexpressing cells (MCF-7/COX-2), generated by transfecting COX-2-encoding plasmids into the poorly invasive MCF-7 breast cancer cells, were more invasive and produced higher IL-8 levels than the parental cells. To investigate the role of IL-8 in COX-2-mediated invasion, MCF-7 parental cells were incubated with IL-8. Exogenous IL-8 increased the invasiveness of MCF-7 cells. IL-8 is one pathway by which COX-2 mediates breast cancer invasion.

Protein kinase A (PKA) and protein kinase C (PKC) are activated by COX-2 and are involved in IL-8 regulation. Inhibition of PKC, not PKA, decreased IL-8 production and invasion in MCF-7/COX-2 cells. Activation of PKC, not PKA, increased IL-8 production and invasion in MCF-7 cells. Thus, the invasive effects of COX-2 are mediated by PKC, not PKA. Activity of the urokinase-type plasminogen activator (uPA) was increased in MCF-7 cells by COX-2 overexpression or by the addition of a PKC activator or by IL-8. Inhibition of PKC decreased uPA activity in MCF-7/COX-2 cells. Furthermore, inhibition of uPA activity decreased the invasiveness of MCF-7/COX-2 cells, indicating that uPA was essential to COX-2-mediated invasion. Herein we demonstrate for the first time a detailed mechanism by which COX-2 increases breast cancer invasion: the PKC/IL-8/uPA pathway.

Introduction

A number of studies have shown high levels of the cyclooxygenase-2 (COX-2) protein in solid tumors (1-3). In breast cancer, COX-2 expression is a predictor of poor disease-free and overall survival (4-6) and has been implicated as a marker of high metastatic potential. COX-2 expression is higher in metastatic tumors (7) and is associated with lymph node and distant metastasis (8-10). COX-2 increases the invasion of breast cancer cells in vitro (11-13) and in vivo (14-18).

Despite the wealth of evidence indicating the role of COX-2 in breast cancer invasion and metastasis, the mechanisms involved are not well defined. High levels of COX-2 have been correlated with increased expression of vascular endothelial growth factor (12), pro-urokinase type plasminogen activator (pro-uPA; 13), interleukin-11 (17), and interleukin-8 (IL-8; 18) in breast cancer cells. However, whether these factors are essential to COX-2-mediated breast cancer invasion, and the mechanisms by which COX-2 increases the expression of these factors are not known. In this study, we investigated the pathways used by COX-2 to increase the invasion of breast cancer cells. Our unpublished microarray results indicate that higher COX-2 expression was associated with increased IL-8 levels. Given the association of IL-8 with increased breast cancer metastasis (19,20), we focused our study on the role of IL-8 in COX-2-mediated invasion and the mechanisms by
which COX-2 increases IL-8 expression in breast cancer cells. We show that COX-2 uses protein kinase C (PKC) to increase the production of IL-8. COX-2 uses IL-8 to activate uPA, resulting in increased invasion of breast cancer cells. This study demonstrates for the first time that COX-2 uses the PKC/IL-8/uPA pathway to increase invasion of breast cancer cells.

Materials and methods

Reagents. Matrigel was purchased from BD Biosciences (Bedford, MA). Hema-3 was purchased from Fisher Scientific (Middletown, VA). Non-reducing sample buffer, 10X zymogram renaturing buffer, 10X zymogram development buffer, Coomassie Blue-R250, and Coomassie Blue destaining solution were purchased from Bio-Rad Laboratories (Hercules, CA). Amiloride was purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Phorbol myristate acetate (PMA), forskolin, GO6976, and PKA inhibitor 6-22 amide were purchased from EMD Biosciences (La Jolla, CA). IL-8 was purchased from Pepro Tech (Rocky Hill, NJ).

Cell lines. The MCF-7 human breast cancer cell line was obtained from American Type Cell Culture (Manassas, VA). MCF-7/COX-2 cells were generated by stably transfecting plasmids encoding the COX-2 gene into estrogen receptor (ER)-positive MCF-7 cells as previously described (21). The pSG5-COX-2 plasmid, which contains a full-length human COX-2 cDNA in the pSG expression vector (22), was used for COX-2 transfection. MCF-7/COX-2 cells were obtained from individual colonies, and continuously cultured in DMEM/F12 medium containing 5% FBS and 500 μg/ml G418. To ensure that MCF-7/COX-2 cells express high levels of COX-2 protein, Western blots using a mouse monoclonal COX-2 antibody (Cayman Chemical, Ann Arbor, MI) were performed (21). We selected clones 8, 12, and 13 that we had previously reported expressed higher levels of COX-2 than the parental MCF-7 cells (21). IL-8 levels and uPA activity were determined in these three clones, but clone 12 was used for all other experiments.

Collection of conditioned medium supernatants. MCF-7 and MCF-7/COX-2 cells (4x10⁵) were plated in T25 flasks in DMEM/F12 medium supplemented with 10% FBS. The next day, MCF-7/COX-2 cells were treated with 6-22 amide (25, 50 nM) or GO6976 (25, 50 nM) and MCF-7 cells were treated with forskolin (1 μM) or PMA (0.1 μM). The following day, MCF-7/COX-2 cells were trypsinized, resuspended in serum-supplemented medium, and counted. Cells were then washed 3 times with serum-free medium. Cells (4x10⁵ in 500 μl) were added into each transwell insert. MCF-7/COX-2 cells were incubated in the presence of the PKA inhibitor 6-22 amide, the PKC inhibitor GO6976, or the uPA inhibitor amiloride. MCF-7/COX-2 cells were incubated in the absence or presence of the PKA activator forskolin or the PKC activator PMA. MCF-7 cells were pretreated with IL-8 (100 ng/ml) for 24 h. Pretreated cells were harvested and washed as described above and then added into transwell inserts at a density of 4x10⁵ in 500 μl in the presence of IL-8 (100 ng/ml). The lower chambers of the inserts were filled with 2 ml of DMEM/F12 medium supplemented with 5% FBS. After a 72-h incubation, non-invading cells on the upper surface of the filter were removed with cotton swabs. Cells that had passed through the pores onto the lower side of the filter were fixed, stained with Hema-3, and photographed. The invaded cells for each filter were counted in five fields under a light microscope at x40 magnification. The invasive ability of the cells was expressed as the mean number of cells that had invaded to the lower side of the filter. The experiments were performed in triplicate and repeated twice.

Statistical analyses. For statistical analysis of the invasion experiments, the Shapiro-Wilk test was first performed to assess the normality assumption of the data. Given that the data were normally distributed, two-sample t-tests were performed to compare the number of invading cells, IL-8 levels, and uPA activity among the various treatments. All analyses were performed in triplicate and repeated twice.
were performed using SAS statistical software at an overall significance level of 0.05.

Results

**COX-2 increases the invasiveness of breast cancer cells.** COX-2 has been shown to increase the invasiveness of MCF-7 breast cancer cells *in vitro* (12). We have previously reported the generation of MCF-7/COX-2 stable transfectants (21). To confirm that COX-2 increases the invasiveness of our MCF-7 breast cancer cells, we randomly selected one clone of MCF-7/COX-2 cells (clone 12) and compared the invasive activity of these cells to that of the parental MCF-7 cells. MCF-7 cells displayed a low invasive capacity on Matrigel (Fig. 1A). MCF-7/COX-2 clone 12 cells were significantly (p<0.05) more invasive than the parental cells (Fig. 1A and B). The number of invaded MCF-7 cells was increased 60% by COX-2 overexpression (Fig. 1B).

**COX-2 uses IL-8 to increase breast cancer cell invasion.** IL-8 production was determined in the concentrated supernatants of MCF-7 and MCF-7/COX-2 clone 12 cells. IL-8 production was increased (p<0.05) by COX-2 overexpression (Fig. 2A). In comparison to the parental cells, MCF-7/COX-2 clone 12 had 5.6-fold higher IL-8 levels (Fig. 2A). To confirm that COX-2 increases IL-8 production, the levels of IL-8 were determined in two other clones of MCF-7/COX-2 cells. Higher levels of IL-8 were also found in the other MCF-7/COX-2 clones; MCF-7/COX-2 clones 8 and 13 had 10.4- and 6.2-fold higher levels of IL-8, respectively, than the parental MCF-7 cells (Fig. 2A). To investigate the role of IL-8 in COX-2-mediated invasion, MCF-7 cells were incubated in the presence or absence of IL-8 in a Matrigel invasion assay. The number of invaded MCF-7 cells was increased (p<0.05) 58% by IL-8 treatment (Fig. 2B and C). These data indicate that at least one pathway by which COX-2 mediates invasion is IL-8 dependent.
PKC activity is involved in COX-2-mediated IL-8 production and invasion in breast cancer cells. COX-2 is known to activate protein kinase A (PKA) and protein kinase C (PKC) pathways (25-28), which have been reported to be involved in IL-8 regulation (29,30). To determine whether COX-2 uses PKA and/or PKC pathways to increase IL-8 production and breast cancer cell invasiveness, MCF-7/COX-2 cells were treated with the PKA inhibitor 6-22 amide or the PKC inhibitor GÖ6976. Conditioned and concentrated supernatants were collected, and IL-8 concentrations in the concentrated supernatants were determined. IL-8 concentrations were expressed as pg/ml/10^6 cells. Values shown are the means ± SD of experiments performed in triplicate. *p<0.05 compared to untreated MCF-7/COX-2 cells.}

Inhibition of PKA activity with 6-22 amide had no significant effect (p>0.05) on the invasiveness of MCF-7/COX-2 cells (Fig. 3B and C). In contrast, inhibition of PKC activity with GÖ6976 decreased (p<0.05) the invasiveness of MCF-7/COX-2 cells in a dose-dependent manner (Fig. 3B and C). The number of invaded MCF-7/COX-2 cells was decreased (p<0.05) 47% and 88% with the 25 and 50 nM concentrations of GÖ6976, respectively (Fig. 3C).

The effects of the PKA activator forskolin or the PKC activator PMA on the production of IL-8 and on the invasiveness of MCF-7 cells were also determined. PMA and forskolin were not cytotoxic to either cell line at the concentrations used (21). IL-8 production was significantly (p<0.05) increased in PMA-treated, but not forskolin-treated, MCF-7 cells (Fig. 4A). IL-8 production was increased 90-fold in MCF-7 cells by PMA treatment (Fig. 4A). The number of
invaded MCF-7 cells was increased by 45% (p<0.05) with PMA treatment, but was not significantly affected by the forskolin treatment (Fig. 4B and C). These data indicate that PKC activity is involved in COX-2-mediated invasion of breast cancer cells.

**COX-2 does not affect MMP-2 and MMP-9 activities in breast cancer cells.** Matrix metalloproteases (MMPs), which are involved in the degradation of the extracellular matrix and basement membranes, are essential to the invasive process. The gelatinases MMP-2 and MMP-9 are frequently detected in breast cancer (31-33) and are associated with metastatic disease (34). To determine whether COX-2 increases the invasiveness of breast cancer cells by increasing MMP activities, gelatin zymography was performed on MCF-7 cells, MCF-7/COX-2 cells, MCF-7 cells treated with IL-8, and MCF-7 cells treated with PMA. MMP-2 activity was detected in MCF-7 cells and was unaffected by COX-2 overexpression or the addition of IL-8 or PMA (Fig. 5A). A very low level of MMP-9 activity was detected in MCF-7 cells and was not altered by COX-2 overexpression or by the addition of IL-8 or PMA (data not shown). These data indicate that alterations in MMP-2 or MMP-9 activities are not involved in COX-2-mediated invasion of breast cancer cells.

**uPA is a downstream mediator of the invasive effects of COX-2/PKC/IL-8 pathway in breast cancer cells.** Since uPA has been implicated in breast cancer invasion and metastasis (35-37), we determined the effect of COX-2 on this pathway. The activity of uPA was increased (p<0.05) by COX-2 overexpression. When compared to the parental cells, uPA activity was increased 62% in MCF-7/COX-2 clone 12 (Fig. 5B). MCF-7/COX-2 clones 8 (82%) and 13 (71%) also had higher (p<0.05) uPA activity in comparison to the parental cells (data not shown). For MCF-7/COX-2 clone 12 cells, the addition of the PKC inhibitor GÖ6976 decreased (p<0.05) uPA activity in a dose-dependent manner. The activity of uPA in MCF-7/COX-2 cells was decreased (p<0.05) 36 and 55% with the 25 and 50 nM concentrations of GÖ6976, respectively (Fig. 5B). In MCF-7 cells, the addition of the PKC activator PMA or IL-8 increased (p<0.05) uPA activity 64 and 45%, respectively (Fig. 5B).

To further confirm the role of uPA in COX-2-mediated invasion, MCF-7/COX-2 cells were incubated with the uPA inhibitor amiloride in a Matrigel invasion assay. Treatment with amiloride decreased (p<0.05) the invasiveness of MCF-7/COX-2 cells in a dose-dependent manner (Fig. 5C and D). The invasion of MCF-7/COX-2 cells was inhibited (p<0.05) 21, 53, and 74% with the 25, 50, and 100 μM concentrations of amiloride, respectively (Fig. 5D). These data indicate that uPA is an important downstream mediator of the invasive effects of COX-2.

**Discussion**

COX-2 is associated with high metastatic potential in breast tumors (7-10) and has been shown to increase invasion and metastasis in vitro and in vivo (11-16). However, little is known regarding the pathways involved in COX-2-mediated invasion. In this study, we showed that transfection of COX-2 into the poorly invasive MCF-7 breast cancer cell line (MCF-7/COX-2) increased invasion in vitro. Prosperi et al (12) also generated stable MCF-7/COX-2 transfectants, which were found to be more invasive than the parental cells. COX-2-transfected MDA-MB-231 cells were also more invasive in comparison to the parental cells (13).

Significantly higher expression of IL-8 and its receptors have been found in breast tumors than in benign breast tissue (38,39). IL-8 has been implicated in promoting breast cancer metastasis. Serum levels of IL-8 are increased in patients with metastatic breast cancer and are associated with an accelerated clinical course, a higher tumor load, and the presence of liver and lymph node involvement (20). IL-8 also increases the invasion of breast cancer cells in vitro (40,41) and has been...
correlated with breast cancer metastasis to lung (42) and to bone in vivo (18, 19). In the present study, we found that COX-2 overexpression increased IL-8 production by ER-positive MCF-7 breast cancer cells. ER is known to decrease IL-8 expression in breast tumors and in breast cancer cell lines (40, 41). It is possible that during the transfection process, MCF-7/COX-2 clones with aberrant ER signaling may have been selected. To overcome this we demonstrated increased IL-8 production in three different MCF-7/COX-2 clones. However, we cannot rule out the possibility that the increase in IL-8 in COX-2-transfected MCF-7 cells is not due to a loss of ER signaling. In this regard, we have previously reported that MCF-7/COX-2 cells were less sensitive to the selective ER modulator tamoxifen (21). Singh et al (18) found that COX-2 expression induced IL-8 production in ER-negative breast cancer cell lines, but not in MCF-7 cells. The difference in our findings from that of Singh et al (18) is not clear; it may be due to different MCF-7 cell clones used. Similar to Prosperi et al (12), we were able to generate stable MCF-7/COX-2 transfectants. However, Singh et al (18) were unable to generate stable MCF-7/COX-2 transfectants; instead they had to use the inducible Tet-On system to generate COX-2 transfectants in MCF-7 cells.

The overexpression and increased activity of PKA and PKC have been implicated in breast tumor development and progression (43, 44). COX-2 is known to activate PKA and PKC pathways (25-28). Furthermore, these pathways have been reported to be involved in IL-8 regulation (29, 30). In the present study, PKC, but not PKA, activation was involved in the COX-2-mediated invasion of MCF-7 breast cancer cells. This contrasts with COX-2-mediated tamoxifen resistance, in which COX-2 uses both PKA and PKC pathways (21).

MMPs and uPA have been implicated in tumor invasion and metastasis. COX-2 has been positively correlated with MMP-2 expression in breast tumor samples (45); however,
the association of COX-2 with MMP-2 activity was not determined in this study. We found that COX-2 did not affect the activity of MMP-2 or MMP-9 in MCF-7 breast cancer cells. Similarly, Takahashi et al (46) found that COX-2 did not increase MMP-2 activity unless the cells were treated with concanavalin A. Concanavalin A is an inducer of membrane-type 1 MMP that processes MMP-2 to convert it to its active form (47).

Increased uPA activity in breast tumors has been correlated with a high rate of relapse and shorter disease-free and overall survival (48-51). Inhibition of uPA has been shown to suppress the invasive and metastatic capacity of breast cancer cells in vitro and in vivo (35-37). Activation of PKC has been correlated with enhanced uPA activity in MCF-7 breast cancer cells (52); however, the mechanisms by which PKC increases uPA activity were not known. Herein we showed that COX-2 uses PKC to increase uPA activity indirectly by increasing IL-8 levels in MCF-7 breast cancer cells. The expression of pro-uPA was significantly higher in COX-2-transfected MDA-MB-231 cells and was correlated with their greater invasiveness in comparison to parental cells (13). While this study suggests that more pro-uPA may be available to bind to its high-affinity cell surface receptor, it does not demonstrate that COX-2 increases uPA enzymatic activity, or that uPA activity is vital to COX-2-mediated invasion. We demonstrated that COX-2 overexpression increases uPA activity in breast cancer cells and that uPA activity is essential to COX-2-mediated invasion.

To our knowledge, we are the first to demonstrate a detailed mechanism by which COX-2 increases the invasiveness of breast cancer cells: the PKC/IL-8/uPA pathway.

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