Prostaglandin E Receptor EP4 Antagonism Inhibits Breast Cancer Metastasis

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Abstract

Cyclooxygenase-2 (COX-2) expression in epithelial tumors is frequently associated with a poor prognosis. In a murine model of metastatic breast cancer, we showed that COX-2 inhibition is associated with decreased metastatic capacity. The COX-2 product, prostaglandin E2 (PGE2), acts through a family of G protein–coupled receptors designated EP1-4 that mediate intracellular signaling by multiple pathways. We characterized EP receptor expression on three murine mammary tumor cell lines and show that all four EP isoforms were detected in each cell. Stimulation of cells with either PGE2 or the selective EP4/EP2 agonist PGE1-OH resulted in increased intracellular cyclic AMP and this response was inhibited with either EP2 or EP4 antagonists. Nothing is known about the function of EP receptors in tumor metastasis. We tested the hypothesis that the prevention of EP receptor signaling would, like inhibition of PGE2 synthesis, inhibit tumor metastasis. Our results show for the first time that antagonism of the EP4 receptor with either AH23848 or ONO-AE3-240, and this chemotactic response was blocked by EP receptor antagonists. Likewise, the proliferation of tumor cells was also directly inhibited by antagonists of either EP4 or EP1/EP2. These studies support the hypothesis that EP receptor antagonists may be an alternative approach to the use of COX inhibitors to prevent tumor metastasis. (Cancer Res 2006; 66(6): 2923-7)

Introduction

Cyclooxygenase-2 (COX-2) is highly expressed in a subset of breast cancers and other malignancies and is associated with a poor prognosis (1). Epidemiologic, preclinical, and clinical studies support a protective role for nonsteroidal anti-inflammatory drugs that target COX (2, 3). We have shown that, in a murine model of metastatic breast cancer, high COX-2 expression and enzymatic activity are associated with more tumorigenic and metastatic behavior (4–6). Although many preclinical studies indicate that COX-2 is a promising therapeutic target in breast cancers and other malignancies, recent concerns regarding the safety of selective COX inhibitors (7) prompted us to seek alternative means to target this pathway. The activities of prostaglandin E2 (PGE2) are mediated by a family of G protein–coupled receptors that are linked to diverse intracellular signaling pathways (8). We hypothesized that, just as blocking PGE2 synthesis by COX inhibitors reduces tumor metastasis (4–6), blocking EP receptor signaling on tumor cells would also inhibit tumor metastasis. We had previously described the presence of receptors for PGE2 on mammary tumor cells (9), however, at the time, it was not known that multiple EP isoforms existed. In the present study, we have characterized the expression of the known EP isoforms on several closely related murine mammary tumor cells and determined the role of one EP receptor in tumor metastasis. We now report that murine and human breast cancer cell lines express all four EP receptor subtypes and that antagonism of EP4 inhibits experimental metastasis.

Materials and Methods

Cell lines and tumors. Murine mammary tumor cell lines 410.4, 410, and 66.1 are maintained as described previously (4–6). For experimental metastasis assays, 3 × 10^6 tumor cells were injected into the lateral tail vein of syngeneic immune competent female BALB/cByJ mice (The Jackson Laboratory, Bar Harbor, ME) and 21 days later, mice were sacrificed and pulmonary metastases were quantified.

Cox inhibitor treatments. The dual COX inhibitor indomethacin and the EP4 antagonist, AH23848, were purchased from Sigma Chemical Co. (St. Louis, MO). AH6809 was purchased from Cayman Chemicals (Ann Arbor, MI). ONO-AE3-208 and ONO-AE3-240 were generous gifts from ONO Pharmaceutical Co. (Osaka, Japan). Line 410.4 or 66.1 cells were cultured in the presence of indomethacin (1.0 μmol/L) or ethyl alcohol as control; the EP antagonists AH23848 (5 μmol/L), ONO-AE3-208 (10 nmol/L), ONO-AE3-240 (5 nmol/L), or DMSO for 48 hours, washed, and 3 × 10^5 viable tumor cells were injected into the tail vein of untreated mice.

Reverse transcription-PCR. RNA was extracted, reverse transcribed and amplified using EP-specific primers. Initial denaturation at 94°C for 2 minutes, followed by 36 cycles of 94°C for 30 seconds, 60°C for 35 seconds, 72°C for 45 seconds, and a 10-minute extension at 72°C. Primers for EP1: forward primer, 5′-GAGGATCAAGTTGTTCAAC- GATGGTC-3′ and reverse primer, 5′-TGAATCCTAAGCTGCTCTATT-TAC-3′; EP2: forward primer, 5′-CTAAGACCGTGTTAAGCTTTC-3′, reverse primer, 5′-ATGGTCACAGTGAAAGTCCATTAAG-3′; EP3: forward primer, 5′-ATACAGTACACAGAGTGTGTCG-3′, reverse primer, 5′-AA- CAGCTTACAGTTAAGCTTTC-3′; EP4: forward primer, 5′-GTCTCAC- TAAAGCATGAAATGTGAAT-3′; EP4-AF: forward primer, 5′-AGTCTCAG- TAAAGCATGAAATGTGAAT-3′; EP4-AR: reverse primer, 5′-ATACAGTACACAGAGTGTGTCG-3′.

Western blotting. Cell lysates prepared and immunoblotting carried out by standard methods using polyclonal rabbit antibodies to murine EP receptors (Cayman Chemicals), followed by horseradish peroxidase–conjugated secondary antibody (Amersham, Piscataway, NJ).

Cyclic AMP assay. Cells were pretreated with indomethacin (1.0 μmol/L) for 24 hours and transferred to complete cell culture medium containing 3-isobutyl-1-methylxanthine (100 μmol/L, Sigma Chemical). Agonists PGE2, PGE1-OH or butaprost and antagonists AH6809, AH23848, ONO-AE3-240, or ONO-AE3-208 were added to cells and incubated for 15 minutes, cell
lysates were prepared, and intracellular cyclic AMP (cAMP) levels were determined according to the manufacturer's instructions using the cAMP biotrak EIA system (Amersham).

Migration assay. Tumor cells, labeled with calcein AM (Molecular Probes, Eugene, OR), and pretreated with indomethacin (1.0 μmol/L) were placed in the upper well of modified Boyden chambers containing nucloepore polycarbonate membranes (8 μm) coated with a mixture of collagen I and fibronectin. PGE2 at various concentrations, base medium or, as a positive control, 2% fetal bovine serum (FBS) were placed in the bottom chamber. EP antagonists (SC19220, AH6809, and AH23848) were placed in the upper chamber. Migration was assessed 24 hours later after removing nonmigrating cells from the upper chamber using a Cytofluor 4000 plate reader measuring fluorescence at 485 nm. Results expressed as percentage-specific chemotaxis (corrected for baseline chemotaxis in the absence of stimulant) of triplicate wells.

Flow cytometry. Tumor cells were prepared in ice-cold 70% ethyl alcohol, blocked with 1% FBS and reacted with polyclonal antibodies to EP1, EP2, EP3, or EP4 (Cayman Chemicals), followed by FITC-conjugated goat anti-rabbit IgG (KPL Inc., Gaithersburg, MD). Fluorescence analyzed by FACScan flow cytometry in the Flow Cytometry Facility of the University of Maryland Greenebaum Cancer Center.

Proliferation assay. Tumor cells were plated in 12-well plates, and the next day, receptor agonists at concentrations ranging from 0.01 to 3.0 μg/mL were added to some wells. Forty-eight hours later, proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay in triplicate determinations.

Results and Discussion

We have previously characterized the expression and function of cyclooxygenases in a murine model of metastatic breast cancer (4–6). Those studies show that the production of PGE2, primarily from the COX-2 isofonn, contributes to the tumorigenic and metastatic properties of these tumors. Tumor-secreted PGE2 could affect the function of many other cells in an exocrine manner, however, we considered the possibility that PGE2 could also directly affect tumor cell behavior in an autocrine manner and that these direct effects would be mediated by one or more EP receptors expressed on the tumor cell.

We described the presence of PGE2 binding activity on tumor cells some years ago (9), however, it has recently been determined that there are actually a family of receptors that bind PGE2 (8). The cellular effects of PGE2 are mediated through a family of G protein–coupled receptors designated EP1, EP2, EP3, and EP4 (8). We have now characterized the expression of all four EP isoforns in three murine mammary tumor cell lines. Line 410 is weakly tumorigenic and nonmetastatic when transplanted to syngeneic BALB/cByJ

elevation in cAMP stimulated by PGE2 or PGE1-OH consistent with the EP4 antagonists AH23848 or ONO-AE3-208 prevented the can stimulate cAMP formation. The EP1/EP2 antagonist AH6809 or Figure 2 shows that treatment of line 66.1 cells with these agonists (EP4/EP2 agonist) could induce increases in intracellular cAMP.

Functional EP2 and/or EP4 receptors on these cells. Although PGE1-OH is a relatively selective agonist for EP4, inhibition of cAMP synthesis, might have therapeutic effects similar to those observed when PGE2 synthesis is prevented with COX inhibitors. Timoshenko et al. (18) described the expression of EP receptors in several murine and human breast tumor cell lines and showed that these cells would migrate in response to PGE2 in vitro. The role of EP receptors in mediating tumor metastasis has not been examined in vivo. We have now compared the ability of EP4 and EP3 antagonists to indomethacin for their antimetastatic activity. To examine the effects of antagonism of tumor-EP in the absence of effects on host EP receptors, line 66.1 or 410.4 tumor cells were treated with indomethacin, AH23848 or ONO-AE3-208 or with the EP3 antagonist ONO-AE3-240 prior to injection into syngeneic BALB/cByJ female mice. Figure 3 shows that a noncytotoxic concentration of indomethacin (1.0 μmol/L) significantly reduces the metastasis of line 66.1, confirming our previous data (5, 6). Likewise, exposure of tumor cells to the EP4 antagonist AH23848 a role in polyp formation in the ApcΔ716 model (12), but other studies have identified EP1 as the critical receptor (13).


Our previous studies have shown that selective or nonselective COX inhibitors are highly effective inhibitors of tumor metastasis (4–6). We have hypothesized that production of PGE2 by tumor cells contributes to metastatic ability in an autocrine fashion in which tumor-PGE2 signals through EP receptors on the tumor cells to enhance tumor dissemination. We further hypothesized that blockade of PGE-mediated signaling, downstream of PGE2 synthesis, might have therapeutic effects similar to those observed when PGE2 synthesis is prevented with COX inhibitors. Timoshenko et al. (18) described the expression of EP receptors in several murine and human breast tumor cell lines and showed that these cells would migrate in response to PGE2 in vitro. The role of EP receptors in mediating tumor metastasis has not been examined in vivo. We have now compared the ability of EP4 and EP3 antagonists to indomethacin for their antimetastatic activity. To examine the effects of antagonism of tumor-EP in the absence of effects on host EP receptors, line 66.1 or 410.4 tumor cells were treated with indomethacin, AH23848 or ONO-AE3-208 or with the EP3 antagonist ONO-AE3-240 prior to injection into syngeneic BALB/cByJ female mice. Figure 3 shows that a noncytotoxic concentration of indomethacin (1.0 μmol/L) significantly reduces the metastasis of line 66.1, confirming our previous data (5, 6). Likewise, exposure of tumor cells to the EP4 antagonist AH23848...
(5.0 μmol/L) also reduced experimental metastasis to a comparable degree. A similar protective effect was observed using a second EP4 antagonist, ONO-AE3-208. In contrast, EP3 antagonism did not affect tumor metastasis. Using a second tumor cell line, we observed that AH23848 was also able to inhibit the metastasis of 410.4 cells. In three experiments, AH23848 reduced metastasis of 410.4 by an average of 30.1 ± 1.5%. To our knowledge, this is the first report that antagonism of EP4 is protective against metastatic disease.

Intracellular signaling via EP receptors can modulate many cellular functions that could affect metastatic capacity. We asked whether EP receptor engagement could modulate an important function of metastatic tumor cells, cellular migration. Using transwell migration assays, we assessed the ability of tumor cells to migrate in response to PGE2 and further determined the effect of EP antagonists on this response. Figure 4A shows that 66.1 tumor cells will migrate in response to PGE2 in a dose-dependent manner. This chemotactic response is inhibited by antagonists of the EP1/EP2 or EP4 receptors (Fig. 4B). These data confirm studies from several laboratories, which showed that tumor cells will migrate in response to PGE2, and that EP2 or EP4 antagonists inhibit this response (18, 19).

We also considered the possibility that EP antagonists directly affect cell growth. Line 410.4 tumor cells were cultured in the presence of AH23848 or AH6809 and cell proliferation was assessed 48 hours later. Both antagonists modestly inhibited cell growth. AH23848, at concentrations from 0.1 to 3.0 μg/mL, inhibited cell growth by 8% to 24% in a dose-dependent manner. AH6809 inhibited growth by 24% or 48% at concentrations of 1.0 or 3.0 μg/mL, respectively. AH23848 has also been reported to inhibit thromboxane receptor–mediated signaling (20), which could contribute to the therapeutic activity observed in the current study.

COX-2 is highly expressed in a subset of human breast tumors and is associated with a poor prognosis (1). Epidemiologic and preclinical data support the therapeutic potential of COX-2 targeting. Recent concerns regarding the potential toxicities of selective COX-2 inhibitors (7) have prompted the search for more effective strategies to exploit the overexpressed COX-2. Antagonism or genetic deletion of individual receptors is protective in models of primary carcinogenesis (11–14, 17). We hypothesized that downstream targeting of the COX-2 product, PGE2, might also prove therapeutic in the metastatic setting. The current results indicate that antagonism of EP4 may be as effective as global COX inhibition in controlling metastatic disease. Future studies will continue to elucidate the mechanisms by which EP4 antagonists are therapeutic, and will investigate the role of other EP receptors in tumor metastasis.

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References

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