Prostaglandin E Receptor EP1 Suppresses Breast Cancer Metastasis and Is Linked to Survival Differences and Cancer Disparities

Xinrong Ma¹, Namita Kundu¹,², Olga B. Ioffe¹,², Olga Goloubeva¹,², Raymond Konger⁴, Claudia Baquet¹,², Phyllis Gimotty⁵, Jocelyn Reader¹, and Amy M. Fulton¹,²,³

Abstract

Cyclooxygenase-2 is frequently overexpressed and associated with poor prognosis in breast cancer. The cyclooxygenase-2 product prostaglandin E₂ (PGE₂) elicits cellular responses through four G-protein-coupled receptors, designated EP1 to EP4, coupled to distinct intracellular signaling pathways. EP4, expressed on malignant breast cells, promotes metastasis; however, a role for EP1 in metastasis has not been investigated. Using a murine model of metastatic breast cancer, we now show that pharmacologic antagonism of EP1 with SC19220 or AH6809 promoted lung colonization of mammary tumor cells by 3.7- to 5.4-fold. Likewise, reducing EP1 gene expression by shRNA also increased metastatic capacity relative to cells transfected with nonsilencing vector but did not affect the size of transplanted tumors. Examination of invasive ductal carcinomas by immunohistochemistry shows that EP1 was detected in both the cytoplasm and nucleus of benign ducts as well as malignant cells in some samples, but was absent or limited to either the nucleus or cytoplasm in other malignant samples. Overall survival for women with tumors that were negative for nuclear EP1 was significantly worse than for women with EP1 expression (P = 0.008). There was no difference in survival for women with differences in cytoplasmic EP1 expression (P = 0.46). Comparing EP1 mRNA in breast tumors from African American and European American women revealed that many more African American breast tumors lacked detectable EP1 mRNA (P = 0.04). These studies support the hypothesis that EP1 functions as a metastasis suppressor and that loss of nuclear EP1 is associated with poorer overall survival and may contribute to disparities in outcome in different populations.

Introduction

In tumors, the cyclooxygenase-2 (COX-2) enzyme is often highly expressed and is associated with poor survival in breast and other malignancies (1). The principal COX-2 product is prostaglandin E₂ (PGE₂). The cellular effects of prostaglandin E₂ (PGE₂) are mediated through a family of G-protein-coupled receptors (GPCR) designated EP1, EP2, EP3, and EP4 (2). Despite structural and sequence similarities among the four EP receptors, they are coupled to different intracellular signaling pathways. Ligand binding of EP1 is associated with Go protein, calcium flux, and subsequent protein kinase C activation, whereas EP2 and EP4 are coupled to Go protein kinase A/adenyl cyclase and mediate elevations in intracellular cAMP. EP4 also activates extracellular signal-regulated kinases 1 and 2 through phosphoinositide 3-kinase (3). Each of the EP receptors has been implicated in primary carcinogenesis (4-8); however, little is known regarding the role of individual EPs in the process of tumor metastasis. We and others have shown that EP4 promotes metastasis in breast, lung, and colon cancer (9-12); however, other EPs, including EP1, had not been investigated. Thorat et al. (13) reported that the nuclear expression of EP1 was associated with lymph node-negative breast cancer. We reported some years ago that EP antagonists, then of unknown specificity, promoted metastasis (14). However, no study had directly examined the role of EP1 in metastasis. We now report that pharmacologic antagonism or gene silencing of EP1 promotes metastasis, leading us to propose that EP1 functions as a metastasis suppressor. Our studies also show that expression of EP1 in the nucleus of malignant cells is associated with significantly better overall survival in women with early-stage disease and hint at a contribution of EP1 to breast cancer disparities.

Materials and Methods

Cells

Line 66.1 and 410 tumor cells were derived from a spontaneously occurring mammary adenocarcinoma in a...
BALB/cByJ female mice were purchased from The Jackson Laboratory. All mice were housed, cared for, and used in strict accordance with the U.S. Department of Agriculture regulations and the NIH Health Guide for the Care and Use of Laboratory Animals. The University of Maryland School of Medicine Animal Facility is fully accredited by the American Association for the Accreditation of Laboratory Animal Care.

**In vivo studies**

Local tumor growth and spontaneous metastases were evaluated by injecting tumor cells s.c. proximal to the left abdominal mammary gland of syngeneic female mice. Tumor diameters were measured with a caliper twice weekly, and mice were euthanized on an individual basis when the diameter reached 16 mm in diameter or earlier if moribund. Lungs were examined for tumor colonies as above.

**Immunohistochemistry**

A tissue microarray was prepared from formalin-fixed, paraffin-embedded tissue specimens acquired through the Breast Cancer Prognostic Study of the Karmanos Cancer Institute, Detroit, MI, and described previously (16). This was a prospective study of 1,306 women with newly diagnosed breast cancer from 1975 to 1983. Clinical and pathologic data were collected from all study subjects. Follow-up data collection from the women continued until 1998. An analysis of all lesions (N = 55) that were classified as invasive ductal carcinoma were used for the current study. For women who died of breast cancer, the mean time to death was 6.1 years. For those alive at last follow-up, the mean follow-up time was 12.4 years. Immunohistochemistry was done using monoclonal EP1 antibody clone 5F12 described in detail in ref. (17) or isotype control antibody. This antibody identifies a protein of the appropriate molecular weight in human or murine tissue or cells transduced with the EP1 gene, but no band is visible in lysates prepared from tissues of EP1–/– mice. Staining was done on a DAKO automatic stainer using diaminobenzidine as the chromagen, and detection was done with the EnVision+ detection system. Staining intensity was scored semiquantitatively separately for the cytoplasm and nucleus, using a scale from 0 to 3: 0, no staining; 1, equivocally positive; 2, definitely positive; 3, intensely positive. Specimens were scored in a blinded fashion by two investigators (N.K. and O.I.).

**Statistical methods**

For studies in mice, the distribution of the number of metastases was compared between the prespecified treatment groups using the nonparametric exact Wilcoxon rank-sum test for groups with a relatively small sample size (<10 mice). The general linear model approach was applied to estimate and compare the average number of lung metastases across larger treatment groups. Square root transformation of the metastasis data was done to decrease variability and to ensure approximate normality. All tests were exact, two-sided, and done at the 0.05 level of significance. In the retrospective cohort study, survival time was defined as time from study entry to death. The censored follow-up time for patients without death information was the date of last contact. EP1 expression for each patient was defined to be the minimum EP1 score of the patients’ multiple tissue microarray cores. Overall survival functions were estimated by the Kaplan-Meier method for 55 patients grouped by staining intensity of nuclear or cytoplasmic EP1. The hazard ratios with the corresponding confidence intervals were estimated using the Cox proportional hazard models. The association of EP1 level and the presence of positive lymph nodes was assessed using the Jonckheere-Terpstra test for doubly ordered rxc contingency table.
Microarray analyses
The preparation and analysis of the gene microarray was previously described in detail (18). Total RNA was extracted from 36 fresh-frozen breast cancers obtained at the University of Maryland Medical System between 1994 and 1999, from 18 African American (AA) and 18 European American (EA) women. Twenty patients had stage II disease, and 16 were diagnosed with stage III disease. RNA was amplified from 20 to 50 μg of total RNA with a T7-based protocol and labeled using random primers and SuperScript reverse transcriptase. Microarray slides containing 8,064 sequence-verified human cDNA clones were prepared, and gene expression was analyzed as previously described. The Lawrence Livermore Laboratory cDNA library was used as a clone source (Invitrogen). Data points representing gene expression ratios were log transformed. To explore the relationship of race to EP1 gene expression, the general linear model was used.

Results
To examine the role of EP1 in modulating tumor behavior, we used a syngeneic murine model of metastatic breast cancer. Lines 410, 66.1, and 410.4 were derived from a spontaneously occurring mammary tumor; the latter two lines are highly metastatic in syngeneic mice. By RT-PCR and Western blotting, we first showed that all three cell lines express EP1 as well as EP2-EP4 (Fig. 1A and B). Others have reported that EP1 is expressed in the nucleus of malignant cells (13, 19). By confocal microscopy, EP1 protein was detected in both the cytoplasm and nucleus of murine mammary tumor cells (Fig. 1C).

A role for EP1 in modulation of metastatic behavior has not been elucidated. To begin to determine a functional role for EP1 in metastasis, we asked if pharmacologic antagonists EP1 (SC19220) or EP1/EP2 (AH6809) could modulate lung-colonizing ability of metastatic cell lines 66.1 and 410.4. In Fig. 2A, line 66.1 cells were cultured in the presence of SC19220 or AH6809 (1.0 μg/mL) for 48 hours, washed, and 2 × 10^5 viable tumor cells were injected into the lateral tail vein of syngeneic female BALB/cByJ mice. On day 18, all mice were euthanized and surface lung tumor colonies were quantified. Pretreatment of line 66.1 tumor cells with either SC19220 or AH6809 increased the number of lung tumor colonies by 3.7- or 5.4-fold, respectively. These results were confirmed using mammary tumor cell line 410.4 pretreated with three different concentrations of SC19220 (1.0, 0.5, and 0.1 μg/mL; Fig. 2B). Although vehicle-treated 410.4 cells are highly metastatic, EP1 antagonism was able to further increase the number of lung tumor colonies. The observation that EP1 antagonism enhances metastatic potential has been replicated in seven independent experiments.

To confirm that blocking EP1 increases metastatic potential, 66.1 cells were engineered with shRNA directed to murine EP1 to stably silence EP1 expression. Line 66.1 cells were transduced with nonsilencing shRNA vector (66.1Ve) or two shRNA sequences targeting EP1 (66.1shEP1), and four stable clones were derived with reduced EP1 expression as determined by quantitative RT-PCR (Fig. 3A). To
determine if changes in EP1 expression affect lung-colonizing ability. 66.1 Ve or 66.1 shEP1 clones were injected i.v. into mice and surface lung tumor colonies were analyzed on day +21. Reduced expression of EP1 was associated with increased capacity to form lung tumor colonies in all four clones (Fig. 3B). To determine if EP1 expression levels affect local tumor growth, 66.1 Ve or three clones expressing reduced levels of EP1 (66.1 shEP1C1, 66.1 shEP1C2, and 66.1 shEP1C3) were implanted proximal to the mammary fat pad of mice and tumor growth was monitored. Regardless of the EP1 expression levels, tumor size was not significantly different in any group (Fig. 3C). Individual animals were euthanized, and lung metastases were quantified as each tumor achieved an average diameter of 16 mm or when mice appeared moribund. Pulmonary metastases were significantly increased ($P < 0.002$) in mice bearing implants of two of three EP1-silenced clones (66.1 shEP1C2, 66.1 shEP1C3) compared with mice bearing implants of 66.1 Ve (Fig. 3D). These data using a spontaneous metastasis model are consistent with the data obtained using pharmacologic EP antagonists or a lung colonization model in which reduced EP1 function resulted in more lung tumor colonies.

We have previously reported that mammary tumor cells also express EP4 but that this receptor plays a very different role in metastasis. Either pharmacologic antagonism or genetic silencing of EP4 leads to reduced, rather than heightened, metastatic capacity (9-11). In the current study, we pooled the data from three to six additional independent experiments to further illustrate this point. Pharmacologic antagonism of EP4 with AH23848 inhibited lung colonization of 66.1 cells by an average of 38 ± 4% in three independent experiments. Likewise, gene silencing of EP4 reduced metastatic potential by an average of 60 ± 6% in six separate experiments. Thus, EP1 and EP4 have dramatically different roles in tumor metastasis. EP4 promotes metastasis. Based on the ability of EP1 to inhibit metastasis in the absence of effects on the primary tumor size, we propose that EP1 acts selectively as a metastasis suppressor.

Limited information regarding the expression or function of EP1 in clinical breast cancer is available. The only reported finding is that EP1 is detected in both the cytoplasm and nucleus of primary human breast tumors by immunohistochemistry and that EP1 expression in the nucleus of malignant cells correlates with good prognostic markers of progesterone expression and lymph node–negative status (13). We investigated the relationship between EP1 expression levels and survival in 54 invasive ductal carcinomas from women who had participated in the Breast Cancer Prognostic Study, a prospective study of 1,306 women newly diagnosed with breast cancer from 1975 to 1983 carried out at the Karmanos Cancer Institute, Detroit, MI. A tissue microarray was prepared that contained one to three tumor samples from each specimen. The tissue array was stained with antibody to human EP1 or isotype-control antibody. Two blinded investigators evaluated the degree of cytoplasmic and nuclear epithelial EP1 staining on a scale of 0 (no positive cells) to 3+ (>75% positive cells). We saw marked heterogeneity both in the degree of staining and in the subcellular localization. Figure 4A shows representative samples of the different patterns observed. EP1 protein is indicated by brown staining, whereas nuclei are identified by the blue counterstain. Figure 4Ai is an invasive ductal carcinoma with no detectable EP1 in either the nucleus or cytoplasm. Figure 4Aii shows benign ducts with prominent brown cytoplasmic as well as brown nuclear EP1 staining obscuring the blue nucleus (ii), an IDC displaying primarily nuclear EP1 but very weak cytoplasmic EP1 (iii), and an IDC specimen in which no nuclear EP1 was detected but moderate levels of cytoplasmic EP1 were seen (iv). Ten of 55 specimens revealed no EP1 staining in either subcellular compartment; nine patients had tumors in which no nuclear EP1 was detectable and five exhibited exclusively nuclear EP1. The remainder had detectable EP1 (1+, 2+, or 3+) in both subcellular compartments.

Based on our prediction that the absence of EP1 would be associated with the worst prognosis, we compared the minimum EP1 intensity for each patient versus overall survival. We considered the expression levels of nuclear and cytoplasmic staining separately. Because 3+ staining was relatively rare, we grouped the intensity values as negative (0) versus positive (1, 2, or 3+) staining. Figure 4B shows the impact of nuclear EP1 on overall survival estimated by the Kaplan-Meier approach. Survival for women with
EP1-negative tumors is significantly worse than for women with any nuclear EP1 expression \((P = 0.008)\). The hazard ratio of death for women with positive EP1 expression is only 40% of those without EP \((95\% \text{ confidence interval}, 0.20–0.81)\). In contrast, overall survival for women was not affected by the level of cytoplasmic EP1 \((P = 0.46; \text{Fig. 4C})\). Thus, detection of even a minimal level of nuclear EP1 in breast tumors was predictive of significantly better survival. This finding is consistent with a metastasis-suppressor role for EP1 revealed in the murine model shown here as well as the clinical study cited earlier in which EP1 expression was associated with lymph node–negative status \((13)\). We also observed a possible trend toward low EP1 and node-positive disease; however, this relationship was not statistically significant \((P = 0.25)\).

Although breast cancer incidence is lower in AA women compared with EA populations, the overall survival in the former group is worse \((20, 21)\). Even when variables such as access to medical care are eliminated, differences in clinical outcome persist, suggesting that breast cancer is inherently more aggressive in AA women. To begin to determine the basis for the observation that breast cancers are often more aggressive in AA women, we examined the expression of \(\sim 8,000\) genes in breast tumors from 18 AA and 18 EA women \((18)\). We now report that expression levels of COX-2, EP2, EP3, or EP4 were not detectably different in these two populations. In contrast, EP1 mRNA was detected significantly less frequently \((\text{negative value compared with reference standard})\) in AA versus EA tumors \((\text{Fig. 5})\). In stage II or III disease, 30% and 25% of AA tumors, respectively, were positive for EP1 mRNA. In contrast, 60% and 88% of EA stage II and III tumors were EP1 positive. This difference in the two populations was significant \((P = 0.04)\). Microarray data must be interpreted conservatively; however, these findings hint at one factor that should be investigated further and that could contribute to the overall worse outcome in AA women with breast cancer.

**Discussion**

In tumors, the principal COX-2 product is prostaglandin E\(_2\). Cellular effects of PGE\(_2\) are mediated through a family of GPCRs designated EP1, EP2, EP3, and EP4 \((2)\). Despite structural and sequence similarities among the four EP receptors, they have different binding affinities...
for PGE2 (Ki of 20, 12, 0.85, and 1.9 nmol/L for EP1-4, respectively) and are coupled to different intracellular signaling pathways (3). Because EP1 is uniquely coupled to a distinct signaling pathway relative to other EPs, it is not surprising that this receptor functions differently in cancer as well.

The precise role of each EP in malignancy is emerging. In colon carcinogenesis, all four EP receptors have been implicated (5-8). EP1 antagonism prevents chemically induced breast carcinogenesis in a murine model (22). Transgenic mice expressing COX-2 in the mammary gland develop mammary tumors; however, this effect was abrogated in EP2−/− mice, implicating the EP2 receptor in mammary carcinogenesis (23). The aromatase enzyme, which catalyzes estrogen biosynthesis, is positively regulated by PGE2; EP1 and EP2 (expressed on stromal cells) are implicated in some studies (24), whereas EP2 and EP4 are key to the induction of the aromatase promoter in other cells (25). The EP1 receptor is implicated in UVB-induced inflammation and skin tumorigenesis (26), but other skin cancer models support a role for EP2 (27). In contrast to the apparent tumor-promoting role of EP1 in several models of primary tumorigenesis, we saw no effect of EP1 silencing on expansion of transplanted mammary tumors. There are many possible explanations for these differences. We directly silenced EP1 on malignant epithelial cells, but it may be that EP1 expressed on other cells is the critical target in primary skin or mammary gland carcinogenesis. It is also possible that the role of EP1 is very different in early-stage tumorigenesis versus during tumor expansion or very late-stage metastasis.

In addition to affecting early events in tumor formation, we and others have shown that EP receptors also play important roles in late-stage breast tumor progression. Breast and colon tumor cells migrate in response to PGE2, and this response is mediated through EP4 (28, 29). Using pharmacologic antagonists or genetic inhibition of EP4, we showed that EP4 promotes metastasis whereas EP3 antagonists do not affect tumor dissemination (9, 11). The protective effect of EP4 blockade was dependent on functioning natural killer cells in the host. Other laboratories have also shown that EP4 silencing inhibits metastasis in murine models of lung and colon cancer (12). PGE2 induces angiogenesis in a model of prostate cancer through the EP4 and EP2 receptors (30). Cell lines derived from inflammatory breast cancer are stimulated to proliferate and invade through EP4 activation (31).

Although a consensus is developing that EP4 supports tumor growth and progression, very little is known regarding the function of EP1. In the current study, we investigated whether EP1 has the same prometastatic role as EP4. Using both pharmacologic and genetic approaches, we now show that inhibition of EP1 signaling or expression increases rather than decreases metastatic capacity. We were
the first to suggest a role for EP receptors in metastasis and had reported some years ago that the EP1 antagonist SC19220 promoted metastasis (14); however, it was not known then that more than one EP receptor exists or that SC19220 would selectively antagonize EP1. To our knowledge, no other studies have examined the role of EP1 in tumor metastasis. Both SC19220 and AH6809 are widely used to examine the physiology of EP receptors, but conclusions regarding the role of individual EP receptors cannot be based on pharmacologic inhibitors alone. Although SC19220 is relatively selective for EP1 on human tissues, AH6809 inhibits both EP1 and EP2, and it is not clear if the receptor specificity of these antagonists is the same in murine tissues (32). Despite the limitations of pharmacologic antagonists, it is quite clear from our previously published studies using two selective EP4 antagonists as well as EP4 gene silencing that EP1 and EP4 play very different roles in tumor biology. Because our gene-silencing data are consistent with the results obtained using pharmacologic approaches, we think that these data support a role for EP1 as a negative regulator of metastatic potential. Because changes in EP1 expression or function did not affect the expansion of the primary tumor, we propose the novel hypothesis that EP1 functions as a suppressor of metastasis.

It is clear that the absolute levels of EP1 alone do not determine function. Similar amounts of EP1 protein were detected in whole-cell lysates of murine tumor cell lines regardless of metastatic capacity. The subcellular location is also likely to determine function. A recent study compared EP1 expression by immunohistochemistry in normal human breast tissue and primary breast cancers (13). EP1 was detected in both the cytoplasm and nuclear membrane in malignant cells. Nuclear EP1 expression was inversely correlated with lymph node involvement and positively correlated with progesterone receptors. Thus, nuclear expression of EP1 was correlated with better prognostic markers. Similar to that study, we observed cytoplasmic and nuclear EP1 in primary breast cancer. We have extended those findings to now show that the absence of nuclear EP1 is associated with significantly worse overall survival. These data strongly support a protective role for EP1. EP1 has also been detected in nonmelanoma skin cancers (17, 33). Interestingly, well-differentiated squamous cell carcinomas have more detectable EP1 than less differentiated lesions (17), consistent with a protective role for EP1.

GPCRs, including EP receptors, belong to a family of receptors composed of seven transmembrane spanning domains. There is now considerable evidence derived from immunofluorescence, electron microscopy, and radioligand binding studies that perinuclear or nuclear GPCRs, including EP1 and EP3, are functional, and these functions may be distinct from the cell surface receptor function (34-36). For example, plasma membrane–expressed EP3 functions in signal transduction, whereas nuclear EP3 is more directly associated with transcription of a distinct set of genes. Most importantly, our data and that of Thorat et al. (13) report that nuclear expression of EP1, but not cytoplasmic EP1, is associated with lymph node–negative disease and better long-term survival in breast cancer, further supporting a distinct role for nuclear-expressed EP1. Functional EP1 has been detected in the nucleus of cholangiocarcinoma cells and is associated with signal transducer and activator of transcription 3, epidermal growth factor receptor (19), and nuclear calcium (36) signaling. Thus, it is intriguing that nuclear EP1 is most closely associated with differences in survival. We have also detected EP1 on

**FIGURE 5.** RNA was isolated from frozen sections of breast tumors from 18 AA and 18 EA women and probed on an ~8,000 gene chip microarray. Gene expression ratios are plotted for individual patients as EP1 detected at levels greater than the gene reference standard (indicated as zero) or below the reference standard. Numbers in bold indicate the number of patients in each subcategory that are positive or negative for EP1 mRNA expression.
normal, immortalized murine and human mammary epithelial cells; thus, it is not a property unique to malignant cells.

As part of an initial effort to understand possible biological bases for differences in outcome for AA versus EA women with breast cancer, we analyzed the expression of ~8,000 genes by gene chip (18). In the current study, we show that the majority of tumors from EA women (72%) have detectable levels of EP1 mRNA, whereas only 28% of AA women had detectable EP1 mRNA. Although gene array data must be interpreted with caution, this marked difference suggests that EP1 should be examined further as a possible contributor to differences in outcome in these two populations. A larger study, including more annotated specimens from AA women, will be required to explore this relationship further.

We do not know the molecular mechanism by which EP1 could suppress metastasis. In the skin, EP1 supports differentiation (17), and a similar function in breast could result in fewer metastases. Both direct effects on EP1-mediated downstream effectors as well as indirect effects on other EP receptors are possible. One proposed mechanism is by shifting PGE2 binding to EP2 and EP4 (37). EP4 promotes metastasis, and it is possible that antagonism of EP1 could enhance EP4-mediated signaling events leading to the observed increased metastatic potential. It is also possible that, due to the lower binding affinity of EP1 for PGE2, this receptor is rarely activated in the tumor milieu and that the potentially protective effects of EP1 on metastasis are not enabled. Specifying the role of each EP in the context of multiple EPs will be challenging. Future studies will examine the molecular function of cytoplasmic versus nuclear EP1 in breast cancer cells to determine the mechanism by which EP1 suppresses metastasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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