The Malignant Phenotype of Breast Cancer Cells Is Reduced by COX-2 Silencing

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Abstract
The cyclooxygenase (COX) pathway is currently targeted for therapeutic intervention in different cancers. We have previously shown that silencing of COX-2 in the poorly differentiated metastatic breast cell line MDA-MB-231 by RNA interference markedly delayed tumor onset and inhibited metastasis. To understand the functional effects of COX-2 silencing underlying the inhibition of tumor growth and metastasis previously reported, we investigated changes in these cells for a number of cancer-associated phenotypes. Cyclooxygenase-2–silenced cells were less able to acidify tissue culture medium, a response that could partly be attributed to decreased lactate production or export detected by reduced lactate in the medium. Consistent with the significantly reduced transcript levels of hyaluronan synthase 2, an enzyme responsible for the total level of hyaluronan secreted by these cells, COX-2 silencing resulted in lower hyaluronan levels secreted in culture medium. Inhibition of human umbilical vein endothelial cell network association in a coculture assay was also observed in COX-2–silenced cells. These data highlight the functional role of COX-2 in pathways that mediate increased malignancy.

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Introduction
Cyclooxygenase 2 (COX-2) is a major contributor to invasive and metastatic stimuli in prostate, colorectal, and breast tumors [1–6]. Cyclooxygenase 2 products are involved in processes central to tumor growth, invasion, and metastasis including angiogenesis and response to hypoxia [4–7]. We previously demonstrated that extrapulmonary metastasis of MDA-MB-231 cells was dependent on COX-2 and that COX-2–silenced MDA-MB-231 cells had increased levels of angiogenic transcripts such as thrombospondin 1 and reduced levels of prometastatic mRNA such as matrix metalloproteinase 1 [5]. In this study, we have examined the functional effects of COX-2 silencing in MDA-MB-231 cells. We focused on lactate production, extracellular acidosis, extracellular matrix (ECM) composition, and human umbilical vein endothelial cell (HUVEC) network formation in COX-2–silenced cells because, as briefly outlined in the subsequent paragraph, these mechanisms are associated with altered metabolism, invasion, vascularization, and processes that result in increased malignancy.

Increased glycolysis and an acidic extracellular pH are associated with a more malignant phenotype. Increased glycolytic activity is mediated, in part, through the stabilization of hypoxia-inducible factor (HIF), which in turn is thought to actively inhibit the conversion of pyruvate to acetyl CoA [8]. Stabilization of the HIF proteins can also up-regulate the expression of transmembrane proteins responsible for the transport of the excess lactate produced outside the cell [9]. Increased lactate in the tumor microenvironment was found to inhibit the metabolism of cytotoxic T cells [10] suggesting that HIF-facilitated accumulation of lactate in the tumor microenvironment may confer advantages for tumor survival and growth. Extracellular acidosis has been associated with increased secretion of proteolytic enzymes and increased invasion and metastasis [11].

The ECM plays an important role in invasion, angiogenesis, and metastasis [12]. We previously observed that COX-2 silencing affects transcripts associated with the ECM [5] including hyaluronan synthase 2 (HAS2). Hyaluronan maintains tissue integrity and mediates adhesion, migration, and differentiation of cells during inflammation, wound repair, and embryonic development [13,14]. It promotes tumor

Abbreviations: COX-2, cyclooxygenase 2; ECM, extracellular matrix; HAS2, hyaluronan synthase 2; HIF, hypoxia-inducible factor; HUVEC, human umbilical vein endothelial cells; PG, prostaglandin

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cell adhesion to laminin and may facilitate invasion through this mechanism [15]. Inhibition of HAS2 using antisense RNA was also found to reduce tumor formation by MDA-MB-231 cells \textit{in vivo} [16].

Whereas the contribution of the hypoxic response to tumor neo-vasculature formation has been extensively studied, other factors including prostaglandins (the major products of the cyclooxygenase reaction) have been shown to affect angiogenesis. Cyclooxygenase 2 overexpression \textit{in vivo} increased microvascular density in the mammary fat pad indicating that COX-2 plays a role in tumor angiogenesis [4]. Human umbilical vein endothelial cell microtubule or microfilament disruption was also found to elicit an increase in both prostaglandins \(E_2\) and \(I_2\) (PGE2 and PG\(L_2\)) secretion [17] suggesting genesis [4].

Human umbilical vein endothelial cell microtubule or microfilament disruption was also found to elicit an increase in both prostaglandins \(E_2\) and \(I_2\) (PGE2 and PG\(L_2\)) secretion [17] suggesting an association between COX-2 function and HUVEC network formation. Additionally, PGE\(2\) has previously been shown to increase Akt phosphorylation and endothelial cell sprouting in a manner dependent on the presence of endothelial nitric oxide synthase activity [18]. Our investigation of the functional effects of COX-2 silencing in MDA-MB-231 cells, specifically lactate production, extracellular acidosis, hyaluronan production and secretion, and HUVEC network formation, revealed significant differences between parental and COX-2–silenced cells.

Materials and Methods

Cell Culture

All epithelial cells were cultured with RPMI 1640 medium (Sigma-Aldrich, St. Louis, MO) supplemented with 8.25% fetal bovine serum (Sigma-Aldrich) without penicillin or streptomycin. Supernatants were collected at the times indicated. Cells used to isolate total RNA for the microarray experiment were seeded (1 × 10\(^6\)) in 100-mm dishes and allowed to attach overnight. Low-passage HUVEC (Clonetics, Walkersville, MD) were cultured in endothelial growth medium (EGM-2; Lonza, Switzerland) supplemented as described by the manufacturer.

Microarray Analysis

RNA from COX-2–containing (MDA-MB-231) and COX-2–silenced (Clone 2 and Pooled) cells was isolated as described previously [5]. Samples were subjected to agarose formaldehyde electrophoresis to determine the quality of the RNA by comparing the ratios of 18S and 28S RNA species. All samples were run in commercial arrays from Affymetrix (Santa Clara, CA), using Human Genome U133Plus 2.0 GeneChip arrays as described in the Affymetrix Web site (http://www.affymetrix.com). Probe hybridization and analysis was run as suggested by the manufacturer in the aforementioned Web site. These descriptions include all information currently considered under the Minimum Information About a Microarray Experiment supportive guidelines, with which the Johns Hopkins Medical Institutions Microarray Core Facility abides in all its procedures. Expression signals were obtained by Robust Multiarray Analysis [19]. The posterior probabilities of the differential expression of genes between the treatments were estimated with an empirical Bayes method using Gamma-Gamma modeling by the bioconductor \textit{R} package, EBarrays. The criterion of posterior probability \(>0.5\) was used to produce a list of genes significantly altered in COX-2–containing and COX-2–silenced cells. Probeset IDs that were not similarly regulated (within 1.5-fold) between Clone 2 and Pooled cells were discarded because the Clone 2 and Pooled clone cells have both been silenced for COX-2 expression during the same transfection procedure as detailed in Stasinopoulos et al. [5], and their common phenotype \textit{in vivo} is likely to be a result of similar changes in gene expression levels.

Lactate and Extracellular pH Measurements

For extracellular lactate measurements, cells (3 × 10\(^5\)) were plated and media were collected at 24, 48, and 72 hours. Supernatant from two independent experiments was assayed using a lactate assay kit (BioVision, Mountain View, CA) following the instructions given by the manufacturer.

For intracellular lactate measurements, cells were cultured to 80% confluence and water-soluble and lipid extracts were obtained from approximately 2 to 3 × 10\(^7\) cells using the dual-phase extraction method [20]. Cell extracts were resuspended in 0.6-ml deuterated water (D\(_2\)O) for magnetic resonance spectroscopy analysis. Five microliters of 0.75% (w/w) 3-(trimethylsilyl) propionic 2,2,3,3-d\(_4\) acid sodium salt (TMSP) in D\(_2\)O was used as an internal standard. Fully relaxed \(^1\)H nuclear magnetic resonance spectra of the water-soluble extracts were acquired on a Bruker Avance 500 spectrometer (Bruker BioSpin Corp., Billerica, MA) as previously described [20]. Signal integrals of two CH\(_3\) peaks in lactate at ∼1.3 ppm were determined and normalized to cell numbers and compared to the standard. To determine concentrations, peak integration \((I)\) from \(^1\)H spectra for lactate were compared to that of the internal standard TMSP according to the equation:

\[
[\text{lactate}] = A_TMSP \cdot \frac{I_{\text{lactate}}}{I_{\text{TMSP}}} \cdot N_{\text{cell}}
\]

In this equation, [lactate] is the intracellular molar concentration per cell of lactate expressed as mole per cell; \(I_{\text{lactate}}\) and \(I_{\text{TMSP}}\) represent the signal integral of lactate and TMSP, respectively, divided by the number of protons; \(A_TMSP\) is the number of moles of TMSP in the sample; and \(N_{\text{cell}}\) is the cell number.

For extracellular pH measurements, COX-2–containing (MDA-MB-231 and Empty Vector) and COX-2–silenced (Clone 2 and Pooled) cells were seeded (3 × 10\(^5\)) and allowed to attach overnight. Fresh medium was added and supernatants collected from plates at 24, 48, and 72 hours. Results represent four independent experiments, and pH was measured at approximately 30°C using a pH meter (MettlerToledo, Columbus, OH).

Real-time Reverse Transcription–Polymerase Chain Reaction

One-step real-time reverse transcription–polymerase chain reaction (RT-PCR) was performed as previously described [5] using 10 ng of total RNA isolated following the Qiagen RNeasy purification protocol as described by the manufacturer (Qiagen, Valencia, CA). Primer sequences for 18S transcript used as a control were 5’-GGTTGA-TCCGTGCCAGTGAC-3’ and 5’-CCGACACAAGGA ACCATAAC-3’. QuantiTect real-time primers for HAS2 catalog numbers: QT0027510 were purchased from Qiagen’s geneglobe collection. Fold differences were calculated using the 2\(^{-}\Delta\Delta\text{Ct}\) method.

Hyaluronan Assay

Hyaluronan quantification from cell media was performed using the Corgenix (Broomfield, CO) Hyaluronic Acid Quantitative Test Kit essentially as described by the manufacturer with the parental MDA-MB-231 media being diluted 1:100 in assay buffer and the COX-2–silenced cells, Clone 2 and Pooled, diluted 1:10. Results
shown represent values from three independent experiments. Standards were plotted using third-order polynomial regression as suggested by the manufacturer, and a Student’s t test was performed to determine statistical significance.

**Immunoblot analysis**

COX-2–containing and COX-2–silenced cells were seeded at 70% confluence, allowed to attach overnight, then washed in PBS, and lysed using M-PER lysis reagent (Pierce, Rockford, IL) plus protease inhibitors (Sigma-Aldrich) at 1:500 dilution. Eighty micrograms of protein was loaded on 10% gel and subjected to SDS-PAGE using standard protocols, blocked for 1 hour for ROBO1 and CXCR4 and overnight for GAPDH in 5% nonfat dry milk in TBS–0.1% Tween 0.1% Tween 20 and ROBO1 (Invitrogen, Carlsbad, CA) and CXCR4 (BD Biosciences) antibodies overnight and GAPDH antibodies (Sigma-Aldrich) for 1 hour. Membranes were washed in TBS–0.1% Tween 20 and secondary antibodies were added for 1 hour (GE Healthcare, Waukesha, WI) then washed with TBS–0.1% Tween 20 and developed using SuperSignal West Pico (Pierce) chemiluminescence substrate.

**HUVEC–Epithelial Cell Coculturing Assay**

Regular 24-well plates were coated with a paraffin pencil to reduce surface tension, and 200 μl of 8.8 mg/ml ECM gel (Sigma-Aldrich) was layered on the bottom of the plate and allowed to solidify in a tissue culture incubator for 3 hours. Human umbilical vein endothelial cell were trypsinized and stained with CellTracker Red (Invitrogen) for 45 minutes in suspension as suggested by the manufacturer. Human umbilical vein endothelial cell (1 × 10⁶) were then mixed with an equal number of unstained COX-2–containing (parental MDA-MB-231, Empty Vector) and COX-2–silenced (Clone 2 and Pooled) cells and plated on top of the ECM layer, and the cells were returned to a tissue culture incubator. Photographs were taken using a fluorescence-capable inverted microscope (Nikon, Melville, NY) at different time points as indicated.

**Results**

**Silencing of COX-2 Reduces Extracellular Acidification**

Microarray analysis between COX-2–containing and COX-2–silenced cells revealed significant reduction in the levels of transcripts associated with extracellular acidosis and lactate and glucose metabolism in COX-2–silenced Clone 2 and Pooled cells. (A) Fold loss of transcript levels associated with extracellular acidosis and lactate and glucose metabolism in COX-2–silenced Clone 2 and Pooled cells. (B) Cell culture media lactate measurements from COX-2–containing parental MDA-MB-231 and Empty Vector cells and COX-2–silenced Clone 2 and Pooled cells taken at the indicated time point. Results are normalized to MDA-MB-231 medium lactate at each time point. Shown are mean ± SE from four experiments; *P < .05. (C) Intracellular lactate concentration in COX-2–containing parental MDA-MB-231 and Empty Vector cells and COX-2–silenced Clone 2 and Pooled cells measured by ¹H magnetic resonance spectroscopy as described in the Materials and Methods section. Shown are mean ± SE from five experiments. (D) pH measurements of cell culture media from COX-2–containing parental MDA-MB-231 and Empty Vector cells and COX-2–silenced Clone 2 and Pooled cells at the indicated time points. Shown are mean ± SE from four experiments; *P < .05; **P < .005.
involved in the metabolic pathways including the glutamate transporter SLC1A1, the lactate transporter SLC16A7 (or MCT-2), the proton (H+) myo-inositol symporter SLC2A13, the glucose transporter SLC2A1 (GLUT1), and hexokinase II (Figure 1A). To determine the effect of COX-2 silencing on lactate export and extracellular lactate, we collected media from cells at 24, 48, and 72 hours after seeding and assayed lactate in supernatants obtained from these cells (Figure 1B). We observed a 20% to 30% reduction of lactate concentration in cell culture media, suggesting that COX-2–silenced cells had decreased lactate production or export. To measure intracellular lactate, we performed 1H magnetic resonance spectroscopy on cell extracts collected from COX-2–silenced and COX-2–containing cells. Cyclooxygenase 2 silencing resulted in a small reduction of lactate production; however, that reduction was not statistically significant (Figure 1C). Cyclooxygenase 2–silenced (Clone 2 and Pooled) and COX-2–containing (MDA-MB-231 and Empty Vector) cells were seeded to 70% confluence, and the pH of the supernatant was measured at 24, 48, and 72 hours after media change. Our results show that COX-2–silenced cells acidified their medium to a much lesser extent and at a slower rate than COX-2–containing cells (Figure 1D). Consistent with these observations, we routinely observed that confluent COX-2–silenced cells showed more alkaline medium than parental cells as seen by the change of color of the pH indicator phenol red present in regular RPMI 1640 media.

Silencing of COX-2 Reduces Extracellular Hyaluronan Levels
To examine whether COX-2 regulates HAS2 expression, we challenged cells with a natural stimulus of COX-2 production, IL-1β (10 ng/ml). Results from real-time PCR suggested that COX-2–silenced cells have less HAS2 mRNA than parental cells (Clone 2: 3.0-fold loss, Pooled: 1.9-fold loss) after IL-1β stimulation in two independent experiments. Exogenous PGE2 regulated HAS2 mRNA in COX-2–containing and COX-2–silenced cells to a similar extent (data not shown). We then examined the presence of hyaluronan in the medium of COX-2–containing and COX-2–silenced cells. We found that COX-2–silenced cells had a significantly lower amount of secreted hyaluronan in three independent experiments (Figure 2) demonstrating that COX-2 regulates the expression of hyaluronan, an important component of the ECM, in these cells.

Silencing of COX-2 Inhibits HUVEC Network Formation
Microarray analysis between COX-2–containing and COX-2–silenced cells revealed significant reduction in the levels of transcripts involved in angiogenesis and vascular integrity including CXCR4 [5], ROBO1, and MEF2C (Figure 3A). Immunoblot analysis for CXCR4 and ROBO1 confirmed that the expression of these proteins is reduced in COX-2–silenced cells (Figure 3B). We examined the role of COX-2–containing and COX-2–silenced cells in HUVEC network formation in a coculturing assay. Human umbilical vein endothelial cell were mixed and cocultured on ECM gel with parental MDA-MB-231, Empty Vector, Clone 2, and Pooled cells for 8 hours. However, the presence of COX-2–silenced cells caused HUVEC clumping with little protrusion formation and minimal, if any, HUVEC association, indicating that silencing of COX-2 actively perturbed HUVEC network formation.

Discussion
We have previously shown that silencing COX-2 expression in MDA-MB-231 cells reduced orthotopic tumor growth in SCID mice and abolished extrapolmonary metastasis in an experimental model of metastasis [5]. In the functional studies performed here, silencing COX-2 expression in MDA-MB-231 cells resulted in a decrease of lactate production or export, a decrease in medium acidification, a decrease in the secretion of the ECM component hyaluronan, and a reduction of HUVEC network formation.

We observed that extracellular lactate concentration and extracellular acidification were reduced in COX-2–silenced cells. Microarray results from COX-2–containing and COX-2–silenced cells revealed alterations in transcripts regulating lactate (SLC16A7 or MCT2) and glutamate transport (SLC1A1) as well as other glycolysis-related transporters and enzymes involved in cancer progression such as hexokinase II. Whereas MCT2 expression has been previously demonstrated in neurons, high monocarboxylate levels found in the breast possibly require active lactate transport [21]. It is possible that breast tumors with a high inflammatory component use active lactate transporters to increase glycolysis and further acidify the extracellular environment. The decrease of lactate production or export and medium acidification on COX-2 silencing is likely a result of the reduction of prostanoïd synthesis, because lactate transport can be mediated, in part, through a PGE2/lactate exchange mechanism [22]. A second mechanism by which silencing of COX-2 may have altered H+ export is through the Na+/H+ exchanger given recent findings that LPS-induced inflammation was inhibited to a similar extent by a Na+/H+ exchange pump inhibitor and the nonselective COX inhibitor indomethacin in vivo [23].
Figure 3. COX-2–silenced cells inhibit HUVEC tubulogenesis. (A) Ratio of expression of angiogenesis-related transcripts in MDA-MB-231 cells over COX-2–silenced Clone 2 and Pooled cells. (B) Immunoblot analysis for ROBO1, CXCR4, and GAPDH in COX-2 containing parental (231) and empty vector (EV) and COX-2–silenced Clone 2 and Pooled cells. (C) Fluorescent microscopy of live HUVEC cells prestained with CellTracker Red and cocultured with COX-2–containing parental MDA-MB-231 or Empty Vector cells or COX-2–silenced Clone 2 and Pooled cells on Matrigel for 8 hours. (D) Bright field photographs of live HUVEC and COX-2–containing parental MDA-MB-231 or Empty Vector cells or COX-2–silenced Clone 2 and Pooled cells after coculturing on Matrigel for 24 hours. All images shown were taken at 4× using a fluorescence-capable Nikon inverted microscope.
We previously observed that silencing COX-2 in MDA-MB-231 cells modified the expression of several transcripts associated with the ECM to a profile consistent with a less metastatic phenotype in an experimental model of metastasis [5]. Previous microarray analysis [5] as well as RT-PCR data showed that COX-2 reduced mRNA expression of HAS2 in MDA-MB-231 cells resulting in reduced hyaluronan production. Hyaluronan synthase 2–silenced MDA-MB-231 cells were previously shown to have reduced primary tumor formation [16] which may explain the reduced ability of COX-2–silenced MDA-MB-231 cells to form primary tumors [5]. Prostaglandins have previously been shown to regulate hyaluronan synthesis through HAS2 in vascular smooth muscle cells a process linked to atherogenesis [14]. Whereas several factors play a role in cell migration and angiogenesis [24,25], our data are consistent with these earlier studies and suggest that silencing of COX-2 in poorly differentiated MDA-MB-231 breast cancer cells may reduce the hyaluronan-facilitated migration of the growing tumor and the proliferation and migration of vascular smooth muscle endothelial cells required for tumor angiogenesis.

The angiogenic phenotype is typically associated with aggressive and metastatic tumors and COX-2–overexpressing tumors have increased microvascular density [4]. Our finding that COX-2–silenced MDA-MB-231 cells actively inhibited HUVEC network formation when cocultured on an ECM gel adds to a growing body of evidence suggesting that COX-2 plays an important role in angiogenesis [4,18,26], a process essential for primary and metastatic tumor growth. Transcriptome comparisons between COX-2–containing and COX-2–silenced cells revealed changes in several angiogenesis-related transcripts such as CXCR4 and ROBO1. Western blot analyses of CXCR4 and ROBO1 confirmed the reduction of these transcripts at the protein level. CXCR4 is a chemokine receptor important in cancer cell invasion [27] and angiogenesis [28], whereas ROBO1 is a receptor of the leucine-rich repeat protein Slit2 that is important for vascular guidance [29] and is overexpressed in breast cancer patient samples as well as MDA-MB-231 cells [30]. It is thus possible that loss of COX-2 expression and function reduces angiogenic signaling mediated by CXCR4 and ROBO1 in poorly differentiated breast tumors. Additionally, we reported loss in transcript levels of LEF1, a transcription factor of the β-catenin pathway previously associated with the induction of the proangiogenic factor IL-8 [31]. COX-2 silencing resulted in the loss of platelet-derived growth factor D mRNA levels previously shown to cooperate with vascular endothelial growth factor E to promote angiogenesis [32]. COX-2 silencing resulted in the loss of several proangiogenic factors explaining, in part, the inability of HUVEC to form networks and self-associate and the marked reduction in orthotopic tumor growth previously reported [5]. We have previously shown that COX-2–silenced MDA-MB-231 cells had significantly elevated levels of thrombomodulin 1 mRNA [5]. Increased thrombomodulin 1 expression may also contribute to the inhibition of HUVEC network formation observed by Yamachi et al. [33].

In conclusion, COX-2 silencing resulted in the loss of several functional phenotypic characteristics associated with malignancy in poorly differentiated MDA-MB-231 breast cancer cells including extracellular acidification, hyaluronan production, and HUVEC network formation. Cyclooxygenase 2 silencing resulted in the loss of expression of metabolic symporters, HAS2, and several proangiogenic factors. Our data implicate COX-2 as a regulator of processes central to tumor metabolism, angiogenesis, and ECM composition, making it a major contributor to a microenvironment permissive to tumorigenesis, invasion, and metastasis.

References


