INFLAMMATION IN THE TUMOR MICROENVIRONMENT

Hostile physiological environments such as hypoxia and acidic extracellular pH which exist in solid tumors, as well as environments created by conventional therapy such as radiation, chemotherapy, and surgery, may promote invasion and metastasis through inflammatory responses and the formation of eicosanoids. As outlined in the schematic in Figure 1, the characteristic response of living vascularized tissue to injury is inflammation, which induces the formation of eicosanoids. These molecular and phenotypic changes, several of which are mediated by COX-2, approach the complexities of a ‘Gordian Knot.’ We review evidence from our studies and from literature suggesting that cyclooxygenase-2 (COX-2) biology presents a nodal point in cancer biology and an ‘Achilles heel’ of COX-2-dependent tumors.

COX-2 IN BASIC, TRANSLATIONAL, AND CLINICAL BREAST CANCER RESEARCH

COX-1 and COX-2 are cytoplasmic enzymes that convert PLA2-activated free arachidonic acid (AA) from membrane phospholipids to prostaglandins (PGs) and thromboxanes (TXs). One major product of the COX-2-catalyzed reaction is PGE2, an inflammatory mediator that interacts with G-protein coupled receptors and binds to specific intracellular receptors that activate a cyclic AMP response element, a NF-κB binding site, and a B binding site, and a cyclic AMP response element, a NF-κB binding site, and a.
shown some promise (Schonthal et al., 2008). and display anti-tumor and anti-inflammatory properties have inflammatory celecoxib analogs designed to not bind to COX-2 pursued further. Attempts to reduce inflammation using anti-independent anti-tumorigenic effects of coxibs needed to be the cardiotoxicity associated with COX-2 inhibition, the COX-2-_frontiers in pharmacology

its anti-tumorigenic effects (Grosch et al., 2006) and that, given has many COX-2-independent functions that are responsible for nuclear factor for interleukin-6/CCAAT enhancer-binding protein (NF-IL6/C/EBP) sequence (Chun and Subh, 2004). The utility of COX-2 as a target for cancer treatment has been debated for decades and the first clinical trials using COX-2-selective inhibitors for cancer treatment took place in the late 1990s when celecoxib was shown to reduce colon adenomas in patients with familial adenomatous polyposis (FAP; Steinbach et al., 2000). Soon thereafter, celecoxib was shown to reduce polyp formation in sporadic colorectal adenocarcinomas (Arter et al., 2006; Bertagnoli et al., 2006), but with increased risk of death by cardiovascular complications (Bazon et al., 2008; Solomon et al., 2008). As a result, celecoxib use for cancer prevention was limited to FAP patients. Results from clinical trials using celecoxib alone suggested a modest effect of celecoxib in primary breast cancer (Martin et al., 2010). Studies using celecoxib in combination with aromatase inhibitors were either terminated early due to cardiovascular side effects (Falbandry et al., 2009) or showed no significant difference in the response rate with the inclusion of celecoxib (Chow et al., 2008). The cardiovascular side effects observed following prolonged celecoxib administration were attributed to an imbalance of eicosanoid production toward the pro-thrombotic TXA₂ (Austman et al., 2007). The limited responses to celecoxib coupled with the significant cardiovascular side effects have resulted in a significant shift in focus to downstream targets such as PG synthases and receptors. Initially, it was reasoned that celecoxib has many COX-2-independent functions that are responsible for its anti-tumorigenic effects (Grosch et al., 2006) and that, given the cardiotoxicity associated with COX-2 inhibition, the COX-2-independent anti-tumorigenic effects of coxibs needed to be pursued further. Attempts to reduce inflammation using anti-inflammatory celecoxib analogs designed to not bind to COX-2 and display anti-tumor and anti-inflammatory properties have shown some promise (Schonthal et al., 2008). Despite the discordance between the promise of basic studies and the limited clinical benefits of coxibs, several observations favor COX-2 as a target for cancer treatment. First, targeting pathways downstream of COX-2 is likely to dilute the effect of COX-2 inhibition, since the COX reaction is the rate-limiting enzyme of prostanoid formation (Samuelsson et al., 1978). Second, there is compelling evidence obtained by studying the effects of COX-2 using short interfering RNA (siRNA) or using the exogenous supplementation of COX-2 reaction products demonstrating that COX-2 promotes carcinogenesis and metastasis. Such evidence is discussed below and in the references cited. Third, TXA₂ synthase inhibitors given concurrently with COX-2 inhibitors could alleviate the cardiovascular side effects attributed to the inhibition of COX-2 by coxibs. A more detailed review of the risks and rewards of targeting COX-2 in cancer was recently published (Menter et al., 2010). Fourth, the limited benefits of celecoxib in human subjects can be explained by the observation that many of the coxib-associated effects observed in vitro and in vivo are not related to COX-2 inhibition, but to COX-2-independent actions of coxibs (Grosch et al., 2006; Schonthal et al., 2008). Fifth, not all tumors or metastatic processes are COX-2-dependent and the expression of a highly inducible enzyme such as COX-2 does not necessarily suggest critical function in every instance it is observed. Thus the utilization of COX-2 inhibitors, even if they specifically inhibited COX-2 function, would not be beneficial until primary tumors and metastatic processes that had a significant requirement for COX-2 were targeted. It would thus be of clinical benefit to discover biomarkers that reflect the activity of COX-2 in tumors and in the tumor microenvironment.

**COX-2 EXPRESSION AND CLINICAL OUTCOMES IN BREAST CANCER**

Several studies have sought to correlate the expression of COX-2 with existing clinical markers in breast cancer. Recently, a large study (n = 1162) of biomarker expression in ductal carcinoma in situ (DCIS) was published (Kerlikowske et al., 2010) where it was shown that the diagnosis of breast tumors by palpation or the concurrent triple expression of p16/COX-2/Ki67 signified an increased risk of recurrence of invasive breast cancer 8 years following initial diagnosis and lumpectomy. A separate study of 248
cases of breast cancer showed that COX-2 expression was elevated in hormone receptor (HR) negative or human epidermal growth factor receptor 2 (HER2) positive subpopulations and correlated with an activation of the oncogene Akt and with poor survival (Glover et al., 2011). Others, however, demonstrated that COX-2 expression correlates with poor outcomes independently of the expression of established markers of breast cancer (Kim et al., 2012). In addition, COX-2 expression has been demonstrated across all clinically useful categories of breast cancers suggesting that COX-2 expression is not predominantly related to hormone or HER2 receptor status. Further complicating the retrofitting of COX-2 positivity within established breast cancer subtypes is the fact that COX-2 expression and function may originate from non-epithelial cellular components of the microenvironment such as the immune response, or the tissue response to injury. Correlative studies that attempt to stratify the expression of COX-2 within current types of breast cancer would miss the transient influence of microenvironment-derived COX-2. It is our view that the discovery of biomarkers that predict the mechanistic association of breast tumor initiation, progression, and metastasis with COX-2 function, can only be attained by the employment of high-throughput/omics approaches on a variety of constituent and representative cells that are engineered to over- or under-express COX-2. The objective would be to derive tumor-promoting COX-2-associated molecular signatures that can be correlated with aggressive phenotypes in experimental animal models and validated in sample tissue or sera of patients.

**COX-2 INDUCES THE EXPRESSION OF ONCogenes BY CO-OPTING BIOLOGICAL EFFECTORS OF HYPOXIA AND DEVELOPMENT**

Given the pleiotropic effects of COX-2 products during development, physiology and disease we have sought to investigate whether COX-2 represents a Gordian knot or an Achilles heel in breast cancer by utilizing COX-2-specific siRNA in a cell-based model of tumor growth and metastasis (summarized in Figure 2). We have observed increased expression of COX-2, in several, but not all, triple negative human breast cancer cells that were also metastatic (unpublished observations). We silenced COX-2 in the most metastatic breast cancer cells and observed a profound decrease of metastasis and tumor onset in vivo, although cell proliferation rates were unaffected in culture (Stasinopoulos et al., 2007). Interactions between the cancer cell and the tumor microenvironment (TME) following COX-2 silencing became apparent in functional imaging assays that revealed a significant decrease of invasion into reconstituted extracellular matrix (ECM; Stasinopoulos et al., 2007; Shah et al., 2012), an altered interaction between endothelial cells and cancer cells following COX-2 silencing (Stasinopoulos et al., 2008), and a significant alteration in glycolysis, pH, and choline metabolism (Stasinopoulos et al., 2008; Shah et al., 2012). The associations between COX-2 and choline metabolism, glycolysis and pH have identified new functional roles of COX-2 that may reveal new biomarkers and new targets to use in combination with COX-2 targeting.

The activation of several genes that form the adaptive response of cells to hypoxia is mediated through the binding of hypoxia-inducible factor (HIF)-1 to the hypoxia response elements that regulate the transcription of these genes (Maxwell et al., 1997). Under oxygenated conditions HIF-1α is rapidly degraded, but under hypoxic conditions HIF-1α is stabilized (Liu and Semenza, 2007). We examined differences in the hypoxia and inflammation-driven functional activation of HIF-1α in COX-2-expressing and COX-2-silenced cells, and found that COX-2 is important for IL-1β, but not hypoxia-driven, HIF-1α stabilization and induction of HIF-1α target genes (Stasinopoulos et al., 2009). These data imply that PGE2 can employ the transcription factor HIF-1, and the multitude of HIF-1 responsive genes, to promote malignant phenotypes associated with HIF-1 activation such as drug resistance, increased invasion, and altered metabolism (Semenza, 2012; Shay and Simon, 2012) even under well-oxygenated conditions. Choline kinase (Chk), a HIF-1 regulated (Glunde et al., 2008) cytoplasmic enzyme responsible for the phosphorylation of choline to phosphocholine (PC) involved in invasion and metastasis (Glunde et al., 2011), was also down-regulated in COX-2-silenced cells (Shah et al., 2012), suggesting a possible mechanism of regulation of phospholipid metabolism by the COX-2-HIF-1 axis. Our results are compatible with a study showing that IL-1-mediated HIF-1 stabilization via COX-2 upregulation and NF-κB activation in lung and colon cancer cells (Jung et al., 2003). Cancers with a strong inflammatory component will most likely have functional HIF-1α activation even under normoxic conditions; targeting COX-2 could minimize these effects. Several insults to tissue such as reactive oxygen species, ionizing radiation, and physical trauma during surgery are known stimuli for the initiation or exacerbation of the inflammatory response (Molla and Paines, 2007; Rundbaek and Fischer, 2008). Peri-operative administration of the COX-2 inhibitor etodolac is being investigated in clinical trial NCT0062684. Our data support the administration of anti-inflammatory agents immediately following surgery and ionizing radiation treatment of patients to minimize activation of the IL-1β–COX-2–HIF-1α axis of oncogenic signaling. This topic has been extensively discussed elsewhere (Choy and Milas, 2003; Intyay and Simon, 2010). Transcriptome analysis revealed differential expression of genes that control angiogenesis, invasion, and differentiation including the Wnt/β-catenin pathway (Stasinopoulos et al., 2012). These changes will identify candidate reporter elements in the promoter of these genes that can be used to image the induction of COX-2 expression. Loss of COX-2 resulted in the loss of lymphoid enhancer-binding factor-1 (LEF-1) mRNA, and nuclear LEF-1 protein, while exogenous supplementation of PGE2 restored nuclear LEF-1 levels in COX-2-silenced cells (Stasinopoulos et al., 2012). Since LEF-1 is a transcription factor that mediates Wnt signaling during development and disease, these results are consistent with the demonstration that PGE2 can promote non-canonical Wnt signaling by directing the translocation of β-catenin from the cytoplasm to the nucleus of colon cancer cells (Castellone et al., 2005). The induction of LEF-1 and the stabilization of HIF-1α by COX-2 provide additional examples of the co-option of molecular pathways central to the response to injury and development by tumors. In addition to providing important cues regarding the role of COX-2 in breast cancer, transcriptome analysis of COX-2-silenced and COX-2 containing cells has indicated candidate reporter elements...
FIGURE 2 | COX-2, Gordian knot or Achilles heel? (A) Physiological processes with significant COX-2 involvement. (B) COX-2 silencing changes the metabolic profile of breast cancer cells to a less aggressive phenotype. (C) COX-2 silencing reduces the invasiveness of breast cancer cell. (D) COX-2 silencing abolishes the extrapulmonary colonization of metastatic breast cancer cells. (E) Sites of primary tumors with extensive COX-2 involvement.

in the promoters of these genes that can be used to image the function of COX-2 in vivo.

**SILENCING OF COX-2 INHIBITS METASTASIS AND DELAYS TUMOR ONSET OF POORLY DIFFERENTIATED METASTATIC BREAST CANCER CELLS**

Breast cancer cells silenced for the expression of COX-2 using stable expression of short hairpin RNA were less able to invade reconstituted ECM than parental cells in vitro (Stasinopoulos et al., 2007). MDA-MB-231 cells silenced for COX-2 expression showed reduced mRNA expression of several oncogenic markers, including IL-11, a marker for metastasis of breast cancer to bone, the Notch1 receptor ligand JAG1, whose expression is correlated with poor breast cancer prognosis, CXCR4, a receptor involved in cancer cell invasion, and matrix metalloproteinase-1 (MMP-1), a secreted enzyme responsible for the degradation of the stroma during breast cancer cell invasion. Dynamic tracking of invasion and metabolism of COX-2-silenced intact MDA-MB-231 cells, using our magnetic resonance (MR) compatible cell perfusion apparatus, under controlled pH, temperature, and oxygenation over 48 h, revealed significantly reduced levels of total choline, PC, and lactate compared to parental MDA-MB-231 cells (Figure 2B) and reduced invasion (Figure 2C). These changes also correlated with a reduction of Chk levels in COX-2-silenced cells (Shah et al., 2012). The metabolic changes are consistent with a less aggressive phenotype since PC and total choline, as well as Chk, are biomarkers of malignancy (Glunde et al., 2011).

Loss of COX-2 resulted in the significant delay of tumor onset when the cells were injected in the mammary fat pad of severe combined immunodeficient (SCID) mice (Stasinopoulos et al., 2007) consistent with the observation that COX-2 was found to be a part of a gene signature that predicted metastasis of MDA-MB-231
cells to the lung (Gupta et al., 2007). Silencing of COX-2 resulted in the inhibition of metastasis to the lungs of SCID mice after intravenous injection (Stasinopoulos et al., 2007) and Figure 2D. Our results show that COX-2 expression modulates the expression or function of many ECM components, including collagen, glycoproteins (e.g., thrombospondin-1 (THBS-1)), hyaluronan, and membrane proteins. COX-2 silencing resulted in the loss of expression of metabolic enzymes involved in cancer progression such as hexokinase II. (SLC1A1) as well as other glycolysis-related transporters and enzymes involved in cancer progression such as hexokinase II.

CONCLUSION

The answer to the question posed in this title cannot be decided directly, or indirectly through the effects of the COX-2 products in vivo, is ideally compatible with identifying mechanisms employed by COX-2-dependent tumors, and identifying markers of COX-2-promoted angiogenesis and metastasis.

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Upregulation of COX-2 in cancer progression.

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