Non-small Cell Lung Cancer Cyclooxygenase-2-dependent Invasion Is Mediated by CD44*

Elevated tumor cyclooxygenase (COX-2) expression is associated with increased angiogenesis, tumor invasion, and suppression of host immunity. We have previously shown that genetic inhibition of tumor COX-2 expression reverses the immunosuppression induced by non-small cell lung cancer (NSCLC). To assess the impact of COX-2 expression in lung cancer invasiveness, NSCLC cell lines were transduced with a retroviral vector expressing the human COX-2 cDNA in the sense (COX-2-S) and antisense (COX-2-AS) orientations. COX-2-S clones expressed significantly more COX-2 protein, produced 10-fold more prostaglandin E₂, and demonstrated an enhanced invasive capacity compared with control vector-transduced or parental cells. CD44, the cell surface receptor for hyaluronate, was overexpressed in COX-2-S cells, and specific blockade of CD44 significantly decreased tumor cell invasion. In contrast, COX-2-AS clones had a very limited capacity for invasion and showed diminished expression of COX-2. These findings suggest that a COX-2-mediated, CD44-dependent pathway is operative in NSCLC invasion. Because tumor COX-2 expression appears to have a multifaceted role in conferring the malignant phenotype, COX-2 may be an important target for gene or pharmacologic therapy in NSCLC.

Cyclooxygenase (also referred to as prostaglandin endoperoxidase or prostaglandin G/H synthase) is the rate-limiting enzyme for the production of prostaglandins (PGs) and thromboxanes from free arachidonic acid (1). The enzyme is bifunctional, with fatty acid cyclooxygenase (producing PGG₂ from arachidonic acid) and PG hydroperoxidase activities (converting PGG₂ to PGH₂). Two forms of cyclooxygenase (COX) have now been described: a constitutively expressed enzyme, COX-1, present in most cells and tissues, and an inducible isoenzyme, COX-2 (also referred to as PGS-2), expressed in response to cytokines, growth factors, and other stimuli (1–4). COX-2 has been reported to be constitutively overexpressed in a variety of malignancies (4–11); we and others have reported that COX-2 is frequently constitutively elevated in human NSCLC (12–16). Previous studies indicate that overexpression of tumor COX-2 may be important in tumor invasion (17, 18), angiogenesis (19, 20), resistance to apoptosis (21–23), and suppression of host immunity (13, 24). Our current studies focus on the role of tumor COX-2 expression in modulating NSCLC invasion.

Tumor metastasis is a complex series of events in which cells migrate beyond tissue compartments and spread to distant organ sites. Cell surface CD44, the receptor for hyaluronate, has an important role in regulating tumor growth and metastasis because it mediates cellular adhesion to extracellular matrix, which is prerequisite for tumor cell migration (25, 26).

While COX-2 expression has previously been linked to enhanced matrix metalloprotease (MMP) expression and invasion (27), the role of CD44 in this COX-2-induced invasion has not been defined. Here, we report that stable overexpression of COX-2 in NSCLC results in up-regulation of CD44. Furthermore, we demonstrate a CD44-dependent increase in invasion in Matrigel matrix assays. In contrast, abrogation of tumor COX-2 expression results in decreased PGE₂ production, diminished CD44 expression and decreased invasion. This is the first report documenting the critical role of tumor COX-2 expression in the regulation of CD44-dependent invasion by human NSCLC.

**EXPERIMENTAL PROCEDURES**

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The abbreviations used are: PG, prostaglandin; COX, cyclooxygenase; NSCLC, non-small cell lung cancer; MMP, matrix metalloprotease; PCR, polymerase chain reaction; EIA, enzyme immunoassay; IL, interleukin; TGF, transforming growth factor; PAGE, polyacrylamide gel electrophoresis.

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pression and PGE2 production, respectively. For each tumor cell line a high COX-2-expressing and PGE2-producing clone for COX-2-S, and a low COX-2-expressing and PGE2-producing COX-2-AS clone, were identified from a survey of 25 clones. These clones were then expanded for further studies.

Measurement of Prostaglandin E2—Control, COX-2-S, and COX-2-AS cells (A549 and H157) were stimulated with IL-1β (200 units/ml, Genzyme, Cambridge, MA) for 24 h. PGE2 concentration in each group (with or without IL-1β stimulation) were measured by EIA using a PGE2 EIA kit (Cayman Chemical, Ann Arbor, MI) as reported previously (28). All measurements were made in triplicate and repeated in at least three separate experiments.

Western Blot Analysis for COX-2 and CD44 Expression—The cells from each treatment group were lysed at 4 °C for 15 min in lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonil fluoride, and 0.6% Nonidet P-40). The cell lysates were centrifuged at 13,000 rpm for 10 min and the supernatant collected. Total protein was measured with a protein assay reagent (Bio-Rad), and 20 μg of cell lysate protein were separated from each treatment group were lysed at 4 °C for 15 min in lysis buffer. Following separation, the proteins were transferred to Hybond nitrocellulose membranes (Amersham Pharmacia Biotech) and the filters probed with anti-human COX-2 antibody (Cayman Chemical). For CD44 detection, the filters were probed with anti-human CD44 antibody 4A4 (29). The membranes were developed by the ECL chemiluminescence system (Amersham Pharmacia Biotech) and exposed to x-ray film (Fujiﬁlm, Fujı Medical Systems Inc., Stamford, CT). Equal loading of samples was confirmed by probing the membranes with β-actin antibody.

Invasion Assay—To quantiﬁe invasion, the membrane invasion assay was carried out in Matrigel-coated invasion chambers (Becton Dickinson Labware, Franklin Lakes, NJ). Control and COX-2 transfectants were cultured in RPMI 1640 supplemented with 10% fetal bovine serum. Tumor cells in log phase growth were detached by trypsin-EDTA (Mediatech) and resuspended in RPMI 1640 with 0.1% bovine serum albumin. Serum-free A549- and H157-conditioned medium was obtained by incubation of these cells for 24 h. This tumor cell-conditioned medium was added in the lower chamber as a chemoattractant, and the resuspended cells (5 × 105) were plated in the upper chamber. Following 18-h incubation at 37 °C in a humidified 5% CO2 atmosphere, the cells in the upper chamber and on the Matrigel were mechanically removed with a cotton swab. The cells adherent to the outer surface of the membrane were ﬁxed with methanol and stained with hematoxylin/ eosin. The invading cells were examined, counted, and photographed by microscopy (Nikon Labphot-2 Microscope with an attached Spot Digital Camera, A. G. Heinz, Lake Forest, CA) at × 50 magnification. Six ﬁelds were counted per ﬁlter in each group, and the experiment was repeated ﬁve separate times. To assess the role of CD44 in mediating Matrigel invasion, the cells from COX-2-S-expressing clones were plated in the presence of 500 ng of anti-CD44 antibody (The Binding Site, Inc., San Diego, CA) or control mouse IgG (Dako Corp., Carpernteria, CA) and the cell numbers determined as described.

RESULTS

Expression of COX-2 and PGE2 Production in COX-2-transduced NSCLC Cells—To evaluate the role of COX-2 expression in mediating the lung cancer invasiveness, two NSCLC cell lines were stably transduced with a retroviral vector encoding COX-2 and selected for G418 resistance. The COX-2-S clones (A549-S and H157-S) exhibited enhanced constitutive COX-2 protein expression by immunoblot (Fig. 1, A and B). In contrast, COX-2 was not detectable in COX-2-AS clones (Fig. 1, A and B). The COX-2 protein expression in the sense clones was found to be significantly higher (3-fold for H157 and 10-fold for A549 cells), as measured by densitometry, than that of parental or vector-transduced cells (data not shown). Compared with parental and vector-transduced cells, COX-2-S clones (H157-S and A549-S) exhibited a 5–12-fold increase in PGE2 production. In contrast, COX-2-AS clones showed decreased constitutive PGE2 production (Fig. 2, A and B). IL-1β is one of several cytokines known to potently up-regulate COX-2 expression in a variety of cells (30). Consistent with our previous findings (28),
CD44-mediated COX-2-dependent NSCLC Invasion

Fig. 3. COX-2-S-modified NSCLC cells demonstrate enhanced invasive capacity. NSCLC cells (parental (P), empty vector (V), COX-2-S (S), and COX-2-AS (AS) transfectants) were used for Matrigel matrix assay. A, left panel, A549; right panel, H157. Significantly more COX-2-S cells invaded the matrix, while COX-2-AS cells showed decreased invasion compared with parental and vector. Anti-CD44 monoclonal antibody, but not control antibody, blocked the enhanced invasion of COX-2-S cells in both cell lines. B and C, bar graphs representing the number of cells that invaded in each group. A 3-fold increase in number of cells invading the matrix is seen in COX-2-S cells: B, A549 (p < 0.05) and C, H157 (p < 0.05).

DISCUSSION

Mounting evidence from several studies indicates that tumor COX-2 activity has a multifaceted role in conferring the malignant and metastatic phenotypes. The data in the current study implicates COX-2 overexpression as a proximal mediator of CD44-dependent invasion in human NSCLC. We demonstrate that augmenting COX-2 expression leads to increased invasion by NSCLC cells. Importantly, this enhanced invasion is associated with increased CD44 expression, and blocking CD44 abrogates the COX-2-mediated enhanced invasion of NSCLC cell mobilization through Matrigel.

Although multiple genetic alterations are necessary for lung cancer invasion and metastasis, COX-2 may be a central element in orchestrating this process (13–17). Studies indicate that overexpression of COX-2 is associated with apoptosis resistance (21–23, 33), angiogenesis (19, 20, 23, 34), decreased host immunity (13, 24), and enhanced invasion and metastasis (27). We reported previously that COX-2 is overexpressed in human NSCLC and the resultant high-level PGE_2 production-mediated deregulation of host immunity by altering the balance of interleukins 10 and 12 (13). Indeed, specific inhibition of COX-2 led to significant in vivo tumor reduction in murine lung cancer models (24). Recently, other studies have corroborated and expanded on our initial findings documenting the importance of COX-2 expression in lung cancer (12, 14–16, 35, 36). COX-2 activity can be detected throughout the progression of a premalignant lesion to the metastatic phenotype (14). Markedly higher COX-2 expression was observed in lung cancer lymph node metastasis compared with primary adenocar-
both the TGF-β genesis (3, 20). The inhibition of programmed cell death in tumors has been implicated in inhibiting apoptosis (21, 38) and angiogenesis (3, 20). The inhibition of programmed cell death in tumors has been implicated in inhibiting apoptosis (21, 38) and angiogenesis (21). Experimental evidence suggests that ligation of the cell surface matrix adhesion receptor CD44 by anti-CD44 antibody induces cell detachment and triggers apoptosis in a variety of cells (39). Thus, inhibiting anchorage dependence mediated by CD44 may contribute to induction of apoptosis (40).

Overexpression of COX-2 also enhances tumor invasiveness and thus may increase metastatic potential (3, 27). Tumor cell invasion involves the active movement of cells across the extracellular matrix (41). Adhesion to extracellular matrix, a critical initial step in the metastatic process, has been found to be CD44-dependent in several tumors (26, 32, 42, 43). CD44 is a receptor for hyaluronate, a major glycosaminoglycan component of the extracellular matrix. In this capacity, CD44 also serves to induce co-clustering with MMP-9 and can therefore promote MMP-9 activity, tumor invasion, and angiogenesis (25, 44). Our findings indicate that tumor COX-2 overexpression in human NSCLC constitutes an important driving force for CD44 induction. Thus, COX-2 expression may form the basis for an important tumor-induced invasive pathway. The fact that CD44-induced MMP-2 and -9 have the capacity to activate latent TGF-β suggests an autocrine and paracrine pathway in which collagen deposition and further invasion may be enhanced (44, 45). The activation of latent TGF-β also provides an additional pathway for tumor-induced immune suppression (44, 46, 47). In addition, recent studies by Sun et al. (48) demonstrate that hyaluronate fragments have the capacity to up-regulate COX-2 by a CD44-dependent pathway. Thus, hyaluronate itself may serve to further enhance tumor COX-2 and CD44 expression leading to maintenance of the COX-2-dependent invasive phenotype.

Lung cancer is the leading cause of cancer death in men and women in the United States (49). Despite therapeutic efforts, 5-year survival in lung cancer patients is less than 15% (50). Defining new molecular targets will lead to more effective therapeutic strategies. Here we document for the first time a pathway whereby COX-2 overexpression leads to CD44-dependent invasion in NSCLC. These findings suggest that therapies targeting COX-2 may diminish the propensity for invasion and metastases in NSCLC.

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