Cyclooxygenase-2 Induces EP₁- and HER-2/Neu-Dependent Vascular Endothelial Growth Factor-C Up-Regulation: A Novel Mechanism of Lymphangiogenesis in Lung Adenocarcinoma

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ABSTRACT

Cyclooxygenase (COX)-2, the inducible isoform of prostaglandin H synthase, has been implicated in the progression of human lung adenocarcinoma. However, the mechanism underlying COX-2’s effect on tumor progression remains largely unknown. Lymphangiogenesis, the formation of new lymphatic vessels, has recently received considerable attention and become a new frontier of tumor metastasis research. Here, we study the interaction between COX-2 and the lymphangiogenic factor, vascular endothelial growth factor (VEGF)-C, in human lung cancer cells and their implication in patient outcomes. We developed an isopropyl-β-D-thiogalactopyranoside-inducible COX-2 gene expression system in human lung adenocarcinoma CL1.0 cells. We found that VEGF-C gene expression but not VEGF-D was significantly elevated in cells expressing COX-2. COX-2-mediated VEGF-C up-regulation was commonly observed in a broad array of non-small cell lung cancer cell lines. The use of pharmacological inhibitors or activators and genetic inhibition by EP receptor-antisense oligonucleotides revealed that prostaglandin EP1 receptor but not other prostaglandin receptors is involved in COX-2-mediated VEGF-C up-regulation. At the mechanistic level, we found that COX-2 expression or prostaglandin E₂ (PGE₂) treatment could activate the HER-2/Neu tyrosine kinase receptor through the EP₁ receptor-dependent pathway and that this activation was essential for VEGF-C induction. The transactivation of HER-2/Neu by PGE₂ was inhibited by way of blocking the Src kinase signaling using the specific Src family inhibitor, PPI, or transfection with the mutant dominant negative src plasmid. Src kinase was involved in not only the HER-2/Neu transactivation but also the following VEGF-C up-regulation by PGE₂ treatment. In addition, immunohistochemical staining of 59 lung adenocarcinoma specimens showed that COX-2 level was highly correlated with VEGF-C, lymphatic vessels density, and other clinicopathological parameters. Taken together, our results provided evidence that COX-2 up-regulated VEGF-C and promotes lymphangiogenesis in human lung adenocarcinoma via the EP₁/Src/HER-2/Neu signaling pathway.

INTRODUCTION

Tumor invasion and metastasis are the critical steps in determining the aggressive phenotype of human cancers. Mortality in cancer patients principally result from metastatic spread of cancer cells to distant organs (1). Clinical and pathological observations have shown that lymph node involvement is one of the earliest features of metastatic tumors. It strongly implicates that lymphatic vessels constitute an important avenue for dissemination of tumor cells. Insight into the mechanism underlying the distribution of cancer cells to regional lymph nodes or distant organs is a challenging and important issue to date.

Cyclooxygenase (COX-2), the inducible form of the COX enzymes (2), catalyzes synthesis of large amounts of prostaglandins (PGs) with diversified biological activities, and its dysregulation plays a vital role in inflammation, tissue damage, and tumorigenesis (3, 4). COX-2 has been reported to be significantly overexpressed in a variety of human malignancies (5–8). Studies from transgenic animals show that the COX-2 gene is involved in the early stages of the oncogenic process of colorectal tumors (9). However, a great amount of evidence suggests the close association of up-regulation of COX-2 with tumor invasion and metastasis in human colorectal, breast, and lung tumors (10–12). In human lung tumors, COX-2 is predominantly expressed in adenocarcinomas, especially in patients with lymph node metastasis (8). To date, only a handful of COX-2-regulated genes have been identified there, including B-cell lymphoma-2 (13), myeloid cell leukemia-1 (14), vascular endothelial growth factor (VEGF)-A (15), CD44 (16), and metalloproteinases (17). The functions of these genes, at present, do not seem to fully account for the effect of COX-2 on tumor invasion and metastasis. It is tempting to speculate that certain unidentified downstream gene(s) of COX-2 may have a role in tumor metastasis.

Lymphangiogenesis, the formation of new lymphatic vessels, has recently become a new research frontier in tumor metastasis due to the discovery that two major lymphangiogenic factors, VEGF-C and VEGF-D, have been linked to promotion of lymphangiogenesis in animal models (18, 19). In addition, the increase of lymphatic vessels density by VEGF-C greatly facilitates the spreading of tumor cells to lymph nodes in these animals (19). The occurrence of lymphangiogenesis can be detected by use several lymphatic vessel-specific markers such as VEGF receptor (VEGFR)-3, LYVE-1, Prox-1, Podoplanin, and Desmoplin (20–25). The VEGFR-3 is relatively specific expressed on the surface of adult lymphatic endothelium, although it is also detected in other cell types (26). The binding of VEGFR-3 and VEGF-C or VEGF-D stimulates the proliferation and migration of lymphatic endothelial cells through mitogen-activated protein kinase and phosphatidylinositol 3-kinase signaling pathways (27) and that in turn augments the assembly of new lymphatic vessels. The elevation of VEGF-C strongly correlates with the formation of metastases in regional lymph nodes in human thyroid, prostate, gastric, colorectal, breast, and lung carcinomas (28). Immunohistochemical staining of tumor specimens demonstrate that VEGF-C is significantly elevated within the tumor cells (29), suggesting that tumor cells are the critical switchers to generate the VEGF-C and activate the lymphangiogenesis.

In this study, we used the cDNA microarray technique to discover that VEGF-C is one of major downstream genes of COX-2 in human lung adenocarcinoma cells. The up-regulation of VEGF-C by COX-2 is mediated by the EP₁ and HER-2/Neu receptor-dependent pathway. We also present evidence for Src kinase play critical role in HER-2/Neu transactivation and subsequent VEGF-C up-regulation mediated...
by COX-2 and its product, PGE₂. COX-2-mediated VEGF-C up-regulation was also found to correlate with the lymph node metastasis and survival in human lung adenocarcinoma patients.

**MATERIALS AND METHODS**

**Cell Culture.** Human lung adenocarcinoma cell lines (A549, PC14, H322, H1299, and CL5) were obtained from American Type Culture Collection, and CI1.0 cells were established as described previously (30). These cell lines were maintained in RPMI 1640 supplemented 10% fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD) with 2 mM t-glutamine (Life Technologies, Inc.), 100 μg/ml streptomycin, and 100 units/ml penicillin in a humidified 5% CO₂ atmosphere.

**Antibodies and Reagents.** Polyclonal goat antihuman VEGF-C, VEGF-D, and VEGFR-3 antibodies were purchased from R&D Systems (Minneapolis, MN). Polyclonal goat antihuman COX-2, HER-2/Neu, polyclonal rabbit anti-anti-phosphotyrosine antibody (PY-20) were purchased from Santa Cruz Biotechnology. The polyclonal Prox-1 antibody was generated as described previously (31). Isopropyl-β-d-thiogalactopyranoside (IPTG), PGE₂, valeryl salicylate, indomethacin, and NS398 were obtained from Sigma. Celebrex, the COX-2-specific inhibitor was obtained from Pharmacia Co. (Piscataway, NJ). The HER-2/Neu kinase inhibitor (PD153035) and the Src kinase inhibitor (PP1) were purchased from Calbiochem Co. (Darmstadt, Germany). Herceptin (Trastuzumab), the neutralizing antibody against HER-2/Neu, was provided from Dr. Chih-Hsin Yang (National Taiwan University Hospital).

**Cell Transfection and Cell Culture.** Non-small cell lung cancer cells were transfected with COX-2-AS-expressing vectors or pcDNA3 (vector control) overnight. The EP receptor agonists and antagonists: 17-phenyl trinor PGE₂ (EP 1 and EP 3 selective agonist), SC19220 (EP 1 antagonist), and PGE₂–I site of the vector pcDNA3. The EP receptor agonists and antagonists: 17-phenyl trinor PGE₂ (EP 1 and EP 3 selective agonist), butaprost (EP 1 agonist), butaprost (EP 1 agonist), prostaglandin E₁ (PCG₃), 16,17-dimethyl PGE₂ (EP₃ selective agonist), SCI9220 (EP 3 antagonist), and PGE₂ were purchased from Cayman Chemicals (Ann Arbor, MI).

**Generation of CI1.0 Cell Line Expressing IPTG-Inducible COX-2.** CI1.0 cells expressing COX-2 were established by transfection with a COX-2 IPTG-inducible expression vector, pNLR-COX-2. After 48 h of transfection, cells were trypsinized and replated in RPMI 1640 with 10% FCS and 800 μg/ml G418. G418-resistant clones (IPTG-COX-2-CL1.0) were selected and expanded.

**Establishment of A549/COX-2-AS Clones.** A 1.9-kbp cDNA fragment of human COX-2 (generously provided by Dr. Shuangan-En Chuang, National Health Research Institute) was cloned into the NotI site of the vector pcDNA3. Antisense (COX-2-AS)-oriented expression vectors were prepared. A549 cells were transfected with COX-2-AS-expressing vectors or pcDNA3 (vector alone) using transfection reagent FuGENE-6 (from Roche Molecular Biochemicals) as described previously (32). The transfected cells were grown in an atmosphere of 5% CO₂ in air at 37°C in cell culture medium consisting of RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM glutamine (Life Technologies, Inc.). After 48 h of transfection, cells were trypsinized and replated in cell culture medium containing 800 μg/ml G418. G418-resistant clones (A549/COX-2-AS and A549/vector) were selected and then expanded for additional studies.

**Microarray Analysis.** Microarrays containing 9600 cDNA sequences from the Integrated Molecular Analysis of Genome and their Expression Consortium library (Research Genetics, Huntsville, AL) were prepared on nylon membranes by an arraying machine built in-house as described previously (33). Experiments in which a colorimetric detection method was used were performed as described by Hong et al. (34) and Chen et al. (35). The microarray image was digitized by use of a drum scanner (ScanView, Foster City, CA). Image analysis and spot quantification were then done by the mOdification of the Cluster program (written in-house). Gene expression data were processed by use of the protocol and program as described previously (33). Genes were grouped on the basis of expression profiles by the self-organizing maps algorithm, described by Tamayo et al. (36).

**Immunoprecipitation and Western Blot Analysis.** The cellular lysates were prepared as described previously (14). Equal amounts of protein were incubated with specific antibody immobilized onto protein A-Sepharose for 2 h at 4°C with gentle rotation. Beads were washed extensively with lysis buffer, boiled, and microcentrifuged. Proteins were resolved on SDS-PAGE and transferred to nitrocellulose membrane. After blocking, blots were incubated with specific primary antibodies. After washing and incubating with secondary antibodies, immunoreactive proteins were visualized by the enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Where indicated, the membranes were stripped and reprobed with another antibody.

**RNA Isolation and Reverse Transcriptase-PCR.** Total RNA was isolated using RNAzol B according to the manufacturer's instructions. Total RNA (1 μg) was reverse transcribed into single-stranded cDNA with Moloney murine leukemia virus reverse transcriptase and random hexamers (Promega, Madison, WI). Amplification of growth factor cDNAs and β-actin cDNA as an internal control in each reaction was carried out by PCR with the following primer pairs described previously (32). The primer sequences are: VEGF-C, 5'-CAGAGGCTCATGACG-3' (forward) and 5'-GGAGAAGTGGTTCGA-3' (reverse); and VEGF-D, 5'-TGGGTCATCTTCTGCAGG-3' (forward) and 5'-GATGATGATCTGGCGCAG-3' (reverse). EP₁ cDNA PCR primer pairs were designed as previously described by Anthony et al. (37). The primer sequences were as follows: EP₁ cDNA PCR are 5'-CTGTGTGATTCATGTTGTCGC-3' (forward) and 5'-GGTTGGTCGCTTAAGATGGCTGAG-3' (reverse); EP₂ cDNA PCR are 5'-GGCCAGATGCTATCCTTCAGCC-3' (forward) and 5'-CTTGAACAGGGGCAACACTTTCTCTGCC-3' (reverse) and 5'-CTTACAGAGGGAAACCAAGATCC-3' (reverse); and EP₄ cDNA PCR are 5'-TGATGTGGGTCGTTGCGT-3' (forward) and 5'-GAGGACGTTGCGGAGAAT-3' (reverse). Reaction mixture was first denatured at 95°C for 10 min. The PCR condition was 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min for 30 cycles, followed by 72°C for 10 min. PCR products were visualized by ethidium bromide staining after agarose gel electrophoresis.

**EP₁, EP₂, and EP₄ Receptor Phosphorothioate Antisense Oligonucleotides.** The sequence of the EP₁-specific antisense oligonucleotides was 5'-GACAGGGTCATGACG-3' and that of control oligonucleotides was 5'-GACAGGGTCATGACG-3'. The sequence of the EP₂-specific antisense oligonucleotides was 5'-GTTGTCATCTTCTGCAGG-3' and that of control oligonucleotides was 5'-GTTGTCATCTTCTGCAGG-3'. The sequence of the EP₄ receptor-specific antisense oligonucleotides was 5'-AGGTGTGGAGGCTTG-3' and that of control oligonucleotides was 5'-AGGTGTGGAGGCTTG-3'. The sequence of the EP₃ receptor-specific antisense oligonucleotides was 5'-AGGTGTGGAGGCTTG-3' and that of control oligonucleotides was 5'-AGGTGTGGAGGCTTG-3'. The sequence of the EP₃ receptor-specific antisense oligonucleotides was 5'-AGGTGTGGAGGCTTG-3' and that of control oligonucleotides was 5'-AGGTGTGGAGGCTTG-3'.

**Immunohistochemistry and Quantitation of Lymphatic Vessel Density.** After rehydration, sections (5 μm) of paraffin-embedded tissue block were incubated in 3% hydrogen peroxide and then blocked by incubation in 2.5% BSA in PBS. The primary antibodies, polyclonal goat antihuman VEGF-C, polyclonal rabbit antihuman COX-2, polyclonal goat antihuman VEGF-D antibody (Sigma, St. Louis, MO), and the polyclonal rabbit antihuman Prox-1 antibody were applied to the slides at a dilution of 1:50 and incubated at 4°C overnight. After washes in PBS, the samples were treated with biotin-labeled secondary antibody (Sigma) at a dilution of 1:200 for 1 h at room temperature. Detection was performed with an avidin-biotin complex kit (Vector Laboratories, Inc.). The slides will be stained with 3,3'-diaminobenzidine, washed, counterstained with hematoxylin, dehydrated, treated with xylene, and mounted. Intensity of immunohistochemical staining: −, negative; +, focal expression < 5% of cancer tissue; ++, focal expression in 5–20% of cancer tissues; and ++++, diffuse expression > 20% of cancer tissues. The tissue with ++ and ++++ staining of COX-2 or VEGF-C was classified as high expression group and tissue with negative and positive staining was assigned as low expression group. For determination of lymphatic vessel density, we identified the area containing the most lymphatic vessel using a 20× objective, scanned the sections at low magnification (×40; Ref. 38). Lymphatic vessel density was then determined by counting the number of Prox-1-positive vessels in a blinded manner from three areas of the highest vessel density/section at ×200. Three sections/tumor were analyzed. Each stained lumen was regarded as a single countable microvessel.

**Patients and Specimens.** Fifty-nine patients who underwent surgery for lung adenocarcinoma at the National Taiwan University Hospital were included in the study. None of the patients had received neoadjuvant chemotherapy or radiation therapy before surgery. Paraffin-embedded, formalin-fixed surgical specimens were collected for immunohistochemical staining. The
histological classification of adenocarcinoma lung cancer was determined as recommended by the WHO (39). Tumor size, local invasion, lymph node metastasis, and final disease stage were determined as described previously (33). The follow-up period lasted up to 110 months. Patients who died of postoperative complications within 30 days after surgery were excluded from the survival analysis.

Statistical Analysis. The data were presented as the mean ± SD. Student’s t test was used to compare data between two groups. Statistical analyses of clinicopathological data were determined as described previously (33). The survival curves were obtained using the Kaplan-Meier method, and the log-rank test was used to test the difference in relapse time between patients of tumors with low and high expression of COX-2 or VEGF-C, as was the difference in survival. All statistical tests were two-way ANOVA. Ps of <0.05 were considered to be statistically significant.

RESULTS

COX-2 Up-Regulates VEGF-C in Human Lung Cancer Cells. To study the downstream effector genes of COX-2 in human lung cancer, we developed an IPTG-inducible COX-2 gene expression system in human lung adenocarcinoma CL1.0 cells and analyzed the expression of its downstream genes by using the high-throughput cDNA microarray. Under the high stringency conditions used, we found that the lymphangiogenic factor VEGF-C gene was one of the highly expressed (~4.9-fold higher than control) genes in COX-2-transfected cells in response to 5 mM IPTG treatment (data not shown). To confirm the results of cDNA expression array screening, we thus extracted RNA and cell lysates from IPTG-inducible COX-2-transfected CL1.0 cells (IPTG/COX-2-CL1.0) and analyzed the VEGF-C mRNA and protein expression by reverse transcriptase-PCR and Western blotting, respectively. The IPTG/COX-2-CL1.0 cells were obtained by pooling with five independent single clones after G418 selection. Upon IPTG treatment, COX-2 but not COX-1 protein was initially increased at 6 h and peaked at 24 h in IPTG/COX-2-CL1.0 cells (Fig. 1A). Comparably, the level of VEGF-C but not VEGF-D mRNA was clearly elevated at 6 h and peaked at 24 h after IPTG treatment (Fig. 1B). Western blot analysis showed that both precursor Mn 58,000 and mature form Mn 29,000/31,000 VEGF-C proteins were significantly increased in IPTG/COX-2-CL1.0 cells after treatment with IPTG (Fig. 1C), indicating a mature and functionally active VEGF-C protein was simultaneously generated in COX-2-overexpressed cells. Elevated levels of PGE2 production, assessed by ELISA (Fig. 1D), paralleled the increased expression of COX-2 protein. This implies that the exogenously overexpressed COX-2 displayed enzymatic activities in the cell system. In addition, this also indicates a possible role of PGE2 in mediating VEGF-C gene expression. We then exposed CL1.0 cells to PGE2 and examined the expression of VEGF-C gene. As expected, treatment caused the significant elevation of VEGF-C mRNA (Fig. 1E, top panel) and protein (Fig. 1E, bottom panel) in parental CL1.0 cells in a dose-dependent manner.

We additionally examined whether COX-2 regulation of the
VEGF-C gene could be observed in other human lung adenocarcinoma cell lines. Of six cell lines tested, we found a strong correlation existed between the expression levels of COX-2 and VEGF-C protein (Fig. 2A). Consistent to protein levels, cell lines expressing higher levels of COX-2 mRNA also displayed greater abundance of VEGF-C mRNA as determined by quantitative reverse transcriptase-PCR (Fig. 2B). Treatment of A549 cells with COX-2-specific inhibitors, NS398 and celebrex, significantly attenuated the endogenous VEGF-C protein level (Fig. 2C). Significantly, the COX-1-specific inhibitor, valeryl salicylate, failed to affect the level of VEGF-C in A549 cells (Fig. 2C). Indomethacin, a nonselective COX inhibitor, partially reduced the VEGF-C protein level. Genetic inhibition of COX-2 by transfected COX-2 antisense-oriented expression vector significantly decreased VEGF-C protein and mRNA level in A549 cells (Fig. 2D). The above data clearly demonstrate that COX-2 tightly regulates VEGF-C gene expression, as was commonly observed in the different human lung adenocarcinoma cells.

**Involvement of EP1 and HER-2/Neu Receptors in COX-2-Mediated VEGF-C Up-Regulation.** PGs exert their effects through interaction with specific EP1–4 receptors. To ascertain the role of EP1–4 subtype receptors in COX-2-mediated VEGF-C up-regulation, we assessed the distribution of these EP subtype receptors in IPTG/COX-2-CL1.0 cells by reverse transcriptase-PCR analysis. The mRNAs of EP1, EP3, and EP2 subtype receptors could be easily detected in cells without COX-2 induction. In contrast, the level of EP2 mRNA was extremely low under the same experimental conditions (Fig. 3A). Upon IPTG treatment for 12 h, the mRNA level of EP1 subtype receptor was evidently increased (~2.3-fold higher than control), whereas other subtypes EP receptor mRNA remained unchanged (Fig. 3A). Again, a similar induction of EP1 receptor mRNA, but not other subtypes, was observed in CL1.0 cells treated with PGE2 (Fig. 3B). This result suggests that COX-2 expression can specifically up-regulate the EP1 receptor mRNA level in human lung cancer CL1.0 cells. We next asked which EP subtype receptors would be involved in the PGE2-mediated VEGF-C up-regulation. To this end, we treated CL1.0 cells with EP1-specific agonists and examined the expression level of VEGF-C gene. Of these agonists tested, only EP1/EP3-selected receptor agonist, 17-phenyl trinor PGE2, significantly induced the increase in VEGF-C mRNA and protein in CL1.0 cells (Fig. 3C). In contrast, butaprost (EP2 agonist) and sulprostone (EP4 agonist) had a slight effect on VEGF-C expression and 16,16-dimethy PGE2 (EP4 selective agonist) failed to up-regulate VEGF-C gene expression. However, the failure of VEGF-C induction by EP2–4 agonists was not attributable to low concentration because similar results were observed even when extremely high doses of EP2–4 agonists used (data not shown). Supportively, treatment of CL1.0 cells with EP1 receptor antagonist SC19220 effectively inhibited PGE2-induced VEGF-C mRNA up-regulation. To determine the role of EP1 receptor-dependent signaling in regulation of VEGF-C expression in CL1.0 cells, specific inhibition of EP1 receptor expression was accomplished with antisense oligonucleotides. As anticipated, the EP1 receptor-specific antisense oligonucleotides used in these studies significantly blocked PGE2-mediated elevated expression of the VEGF-C in non-small cell lung cancer cells (Fig. 3D, top).
Furthermore, treatment with EP$_3$ or EP$_4$ receptor-specific antisense oligonucleotides did not abolish PGE$_2$-mediated up-regulation of VEGF-C expression (Fig. 3). As a result of exceedingly low level of EP$_2$ receptor expression with or without of PGE$_2$ treatment, we did not abolish EP$_2$ receptor expression by EP$_2$ receptor-specific antisense oligonucleotides. Above studies using pharmacological treatment or genetic inhibition clearly demonstrated a critical role of EP$_1$ receptor in the PGE$_2$-mediated up-regulation of VEGF-C.

Our (32) and others' studies (41) have demonstrated that HER-2/Neu overexpression could increase the expression of VEGF-C gene in breast cancer specimens and cell lines. In addition, another study showed that PGE$_2$ could transactivate the epidermal growth factor receptor in human colon cancer cells (17). On the basis of these studies, we tested the hypothesis of possible cross-talk between COX-2-derived prostanoids and HER-2/Neu receptor signaling in our lung cancer cell system. In the IPTG-inducible system, we found that COX-2 expression by treatment with IPTG significantly increased tyrosine phosphorylation of HER-2/Neu receptor (Fig. 4A). NS398 completely abolished this action of COX-2. In parental CL1.0 cells, PGE$_2$ treatment for 30 min also induced an evident tyrosine phosphorylation of HER-2/Neu receptor (Fig. 4B). Treatment with EP$_1$ receptor antagonist SC19220 effectively blocked COX-2 or PGE$_2$-induced tyrosine phosphorylation of HER-2/Neu (Fig. 4, A and B). In addition, treatment with EP$_1$ receptor-specific antisense oligonucleotides, but not other EP receptor-specific antisense oligonucleotides, significantly blocked PGE$_2$-mediated tyrosine phosphorylation of HER-2/Neu (Fig. 4B). These data suggest that EP$_1$ receptor-dependent signaling plays a direct role in PGE$_2$-mediated transactivation of HER-2/Neu receptor. We additionally examined whether this EP$_1$-dependent transactivation of HER-2/Neu by PGE$_2$ could mediate signaling to...
up-regulate VEGF-C gene. Blocking HER-2/Neu activity by Herceptin (a HER-2/Neu blocking antibody) or PD153035 (a HER-2/Neu kinase inhibitor) strongly reduced the level of VEGF-C protein and mRNA in IPTG-inducible COX-2 expression (Fig. 4C, top and bottom panels), as well as in PGE$_2$ treatment models (Fig. 4D, top and bottom panels). Taken together, our study delineates a novel mechanism underlying COX-2 up-regulated VEGF-C gene expression through the EP$_1$ receptor-dependent transactivation of HER-2/Neu receptor.

**Involvement of $[Ca^{2+}]_i$ in PGE$_2$-Induced VEGF-C Up-Regulation.** It has been reported that activation of EP$_1$ augments intracellular calcium mobilization and related downstream signals (40). In our cellular model, $[Ca^{2+}]_i$ was 54 ± 7.07% higher in PGE$_2$-treated cells than control cells (data not shown). We next investigated whether chelating of intracellular Ca$^{2+}$ could abolish the PGE$_2$-induced transactivation of HER-2/Neu. Interestingly, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (acetoxymethyl) ester (BAPTA-AM) (10 µM) significantly abrogated the
PGE₂-induced tyrosine phosphorylation of HER-2/Neu and subsequent VEGF-C induction. This inhibition could be restored by treatment with 0.5 μM thapsigargin, a reagent that increases cytoplasmic free Ca²⁺ concentrations (data not shown).

**PGE₂-Induced HER-2/Neu Transactivation and Subsequent VEGF-C Up-Regulation Required the Src Kinase.** Transactivation is one of cross-talk among different receptors and transactivation of receptor tyrosine kinase (RTK) by G-protein-coupled receptor (GPCR) has been reported in several cell types (42, 43). However, the circumstances under which HER-2/Neu is activated in lung adenocarcinoma cells are poorly understood. In particular, G-protein-coupled receptor-mediated transactivation of RTKs was reported in some recent studies to require the presence of Src family kinases (44, 45). Because cytoplasmic tyrosine kinases of the Src family have been implicated recently in G-protein-coupled receptor-mediated RTK transactivation, we investigated a possible intermediate role of Src between EP₁ and HER-2/Neu. For this purpose, we used the tyrosine kinase inhibitor PP₁, which was reported to be specific for Src-like kinases (46). Exposure of cells to PGE₂ caused an increase in tyrosine phosphorylation of Src, as determined by specific anti-phospho-src antibody (Fig. 5A). Pretreatment of cells with SC19220 significantly attenuated PGE₂-induced tyrosine phosphorylation of Src kinase (Fig. 5A) similar to that noted with PP₁. These data defined that Src was a downstream mediator of EP₁, signaling activated by PGE₂ in CL1.0 cells. Inhibition of Src kinase either by treatment with PP₁ or expression of a kinase-defective Src mutant (dominant negative src, carrying a K-to-D mutation at position 297) strongly reduced the transactivation of HER-2/Neu, which was a critical step of VEGF-C up-regulation in response to PGE₂ (Fig. 5B). Supportively, Src kinase inhibitor PP₁ abolished the transactivation of HER-2/Neu and reduced the up-regulation of VEGF-C protein and mRNA by PGE₂ (Fig. 5C, top and bottom panels). These results indicate that PGE₂ transactivation of HER-2/Neu and the following VEGF-C up-regulation required the Src function.

**COX-2 Expression Correlated with VEGF-C Level, Lymphatic Vessel Density, Lymph Node Metastasis, and Patient Survival in Lung Adenocarcinoma.** Immunohistochemical analysis of 59 lung adenocarcinoma specimens revealed that the VEGF-C expression (negative, 1+, 2+, or 3+ staining) in tumors was significantly associated with the expression of COX-2 (negative, 1+, 2+, or 3+ staining) in tumors (tested by Spearman’s nonparametric correlation test, correlation coefficient = 0.665, P < 0.001; Table 1). The representative immunohistochemical staining patterns of COX-2 and VEGF-C in human lung adenocarcinomas are shown in Fig. 5A. A representative Western blot of COX-2 and VEGF-C expression is shown in Fig. 5C. The correlation between levels of cyclooxygenase-2 (COX-2) and vascular endothelial growth factor-C (VEGF-C) expression in human lung adenocarcinomas is shown in Table 1. The correlation coefficient between COX-2 and VEGF-C expression was calculated using Spearman’s nonparametric correlation test. The correlation coefficient was 0.665 (P < 0.001). The number of tumor samples from patients with lung adenocarcinoma was 59. A representative immunohistochemical staining pattern of COX-2 and VEGF-C is shown in Fig. 5A. A representative Western blot of COX-2 and VEGF-C expression is shown in Fig. 5C.
VEGF-C from consecutive serial sections were near-identical in lung adenocarcinoma tissues implying highly correlated expression (Fig. 6A). We then quantified the number of lymphatic vessels in the COX-2 high and low expression lung adenocarcinoma using antibodies against Prox-1 and VEGFR-3. Immunohistochemical analysis shows that intratumoral lymph vessels were in fact present in carcinomas with a pushing margin (data not shown). These intratumoral lymphatic vessels had a distinctive reticular architecture with numerous tiny or ill-defined lumina that revealed similar distribution pattern staining with antibodies to Prox-1 and VEGFR-3 in the same tumor (Fig. 6B). Quantification of the Prox-1-positive vessels in the tumors indicated that tumor of high expression level of COX-2 had a higher density of tumor lymphatic vessels than in low COX-2 expressing tumors (39.87 ± 8.37 vessels/microscopic field for COX-2 high expression tumors, n = 10, and 17.13 ± 10.12 for COX-2 low expression tumors, n = 10, P = 0.006, Student’s t test, Fig. 6C). There were similar results by quantification of the VEGFR-3-positive vessels, the density in COX-2 high expressing tumors was ~2-fold higher than COX-2 low expressing tumors.

COX-2 high expression patients were more likely than low expression patients to have lymph node metastasis (N1, N2, and N3; P = 0.017). VEGF-C high expression patients were more likely than low expression patients to have advanced disease (stage III or IV; P = 0.004) and lymph node metastasis (P < 0.001; data not shown). For COX-2 low expression patients, the median of probability of survival was 68.8 months, and they had a statistically significantly longer survival than COX-2 high expression patients (median survival = 18.2 ± 5.8 months; 95% confidence interval = 6.9–29.4 months; log-rank test, P = 0.004; Fig. 6D). The median survival time for patients with tumors with high VEGF-C was 13.9 ± 4.6 months; 95% confidence interval = 4.8–22.9 months, significantly shorter than that for patients with tumors with low VEGF-C expression (68.8 months; P < 0.001, log-rank test, Fig. 6E). These results provide a possible explanation that COX-2 high expression patients were more likely than low expression patients to have poor prognosis, possibly resulting from COX-2-derived lymphangiogenesis in human lung adenocarcinoma.

**DISCUSSION**

In the present study, we demonstrate a novel function of COX-2 in tumor lymphangiogenesis by both molecular dissection and clinical inspection. This study demonstrates, for the first time, that COX-2 and its derived prostanoids, i.e., PGE₂, are able to up-regulate VEGF-C gene through EP₁ and HER-2/Neu-dependent pathway. This up-regulation of VEGF-C by COX-2 commonly occurs in a wide variety of non-small cell lung cancer cell lines and involves of the Src kinase. Clinical examination of 59 human lung adenocarcinomas also reflects the close association between COX-2 and VEGF-C. The high expression levels of COX-2 correlated with high VEGF-C level, higher lymphatic vessel density, more lymph node metastasis, and shorter survival rate of patients. From molecular regulation to patient outcomes, we provide a new role of COX-2 enzyme in human lung adenocarcinoma.
COX-2 is a pleiotropic enzyme that mediates many physiological functions such as inhibition of cell apoptosis, augmentation of angiogenesis, as well as increased cell motility. These COX-2-mediated functions are mediated in part by various genes such as B-cell lymphoma-1 (13), myeloid cell leukemia-1 (14), VEGF-A (15), CD44, and matrix metalloproteinases (16). High COX-2 levels in human colon (47) and gastric cancers (48) greatly promote the tumor growth by increasing microvessel density, i.e., angiogenesis. VEGF-A was identified as a major downstream effector gene of COX-2-induced angiogenesis in colon cancer (47). Furthermore, genetic disruption of EP2 but not other PGE2 receptors caused an inhibition of VEGF-A, as well as angiogenesis induced by COX-2 in an intestine tumor model (49). Hansen-Petrik et al. (50) have shown that selective antagonism of EP1 results in fewer tumors in ApoMin/H11001 mice and identified the important role of EP1 in PGE2-induced intestinal tumorigenesis. However, here we demonstrate that the EP1 but not other EP receptors was required for COX-2 or PGE2-induced up-regulation of VEGF-C gene. Although treatment with Butaprost (EP2 agonist) and Sulprostone (EP3 agonist) appears to slightly up-regulated VEGF-C (Fig. 3C, quantification by Edit EZ-1D Software from EZLab Technology Co., Ltd.), this induction was not statistically significant by Student’s t test analysis (P > 0.05, compared with control). Furthermore, we could not inhibit PGE2-induced VEGF-C up-regulation by EP3 or EP4 receptor-specific antisense oligonucleotides (Fig. 3D). We suggest that VEGF-C up-regulation by these two agonists may be as a result of the minor cross-reaction with EP1 receptor-dependent signaling. In addition, the mRNA level of EP1 receptor was elevated by COX-2 overexpression or PGE2 treatment (Fig. 2, A and B). Our current data and other studies suggest that the mechanisms by which COX-2 and its derived PGs regulate VEGF-C and VEGF-A are quite distinct. This also lends support to the hypothesis that angiogenesis and lymphangiogenesis are separately governed by COX-2 activity.

Using Herceptin or EP1 receptor antagonist significantly suppressed COX-2 or PGE2-induced VEGF-C gene up-regulation. These data strongly suggest that EP1 and HER-2/Neu are critical mediators to transduce the signaling of VEGF-C expression and tumor lymphangiogenesis. Indeed, our data and those of others on breast cancer showed that HER-2/Neu overexpression greatly increases the VEGF-C gene expression (32, 41). A related finding made by Pai et al. (17) was that PGE2 transactivates epidermal growth factor receptor and in turn promotes colon cancer growth and gastrointestinal hyperplasia. However, the authors did not clarify which kind of PG receptors are involved in the transactivation of epidermal growth factor receptor. Interestingly, COX-2 has been found to be overexpressed in HER-2/Neu-positive breast cancer (51). Both activator protein-1 and polyomavirus enhancer A-binding protein-3 factors are involved in the transcriptional regulation of COX-2 by HER-2/Neu (52). Combining the above findings, we propose that COX-2 and HER-2/Neu regulate each other in certain tumor cells, forming an amplification loop to facilitate the oncogenesis by activation of downstream effector genes, e.g., VEGF-C.

Our results suggest that PGE2 transactivation of HER-2/Neu and subsequent up-regulation of VEGF-C expression is facilitated by increased[Ca2+]. We also demonstrate that this up-regulation of [Ca2+] may be attributable to induction by PGE2 and its following signaling through EP1. Calcium was also a critical angiogenesis mediator in the hypoxia-induced activation of hypoxia-inducible factor-1 and VEGF expression in HepG2 cells (53). In our cell model,
we present VEGF-C as a novel calcium-related metastatic factor. Moreover, we defined calcium-mediated HER-2/Neu transactivation as another critical positive regulator of VEGF-C expression in CL1.0 cells.

Transactivation of distinct RTKs may contribute in different cellular responses and physiological functions such as proliferation, differentiation, antiapoptosis, and metastasis (46). Src kinase could be physically associated with some RTKs, and Src kinase activity is thought to be important for RTKs-mediated signal transduction (54). Src is known to interact with insulin-like growth factor receptor (55), and in a recent study, Tang et al. (56) showed that activation of insulin-like growth factor receptor increases VEGF-C expression through the phosphatidylinositol 3-kinase-dependent pathway and promotes lymph node metastasis in the animal model. According to these studies, Src kinase may be a modulator in VEGF-C regulation.

In conclusion, our work suggests a cause-effect link between COX-2 overexpression and tumor lymphangiogenesis through VEGF-C activity in lung adenocarcinoma. The up-regulation of VEGF-C by COX-2 through EP3/Src/HER-2/Neu signaling pathway was demonstrated in this study. Our findings open newer therapeutic modalities against lymphangiogenesis and lymphatic bome metastasis by targeting COX-2.

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REFERENCES


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