Tumorigenesis and Neoplastic Progression

COX-2 and Prostaglandin EP3/EP4 Signaling Regulate the Tumor Stromal Proangiogenic Microenvironment via CXCL12-CXCR4 Chemokine Systems

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Bone marrow (BM)–derived hematopoietic cells, which are major components of tumor stroma, determine the tumor microenvironment and regulate tumor phenotypes. Cyclooxygenase (COX)–2 and endogenous prostaglandins are important determinants for tumor growth and tumor-associated angiogenesis; however, their contributions to stromal formation and angiogenesis remain unclear. In this study, we observed that Lewis lung carcinoma cells implanted in wild-type mice formed a tumor mass with extensive stromal formation that was markedly suppressed by COX-2 inhibition, which reduced the recruitment of BM cells. Notably, COX-2 inhibition attenuated CXCL12/CXCR4 expression as well as expression of several other chemokines. Indeed, in a Matrigel model, prostaglandin (PG) E₂ enhanced stromal formation and CXCL12/CXCR4 expression. In addition, a COX-2 inhibitor suppressed stromal formation and reduced expression of CXCL12/CXCR4 and a fibroblast marker (S100A4) in a micropore chamber model. Moreover, stromal formation after tumor implantation was suppressed in EP3^{−/−} mice and EP4^{−/−} mice, in which stromal expression of CXCL12/CXCR4 and S100A4 was reduced. The EP3 or EP4 knock-out suppressed S100A4⁺ fibroblasts, CXCL12⁺, and/or CXCR4⁺ stromal cells as well. Immunofluorescent analyses revealed that CXCL12⁺CXCR4⁺S100A4⁺ fibroblasts mainly comprised stromal cells and most of these were

recruited from the BM. Additionally, either EP3- or EP4-specific agonists stimulated CXCL12 expression by fibroblasts *in vitro*. The present results address the novel activities of COX-2/PGE₂-EP3/EP4 signaling that modulate tumor biology and show that CXCL12/CXCR4 axis may play a crucial role in tumor stromal formation and angiogenesis under the control of prostaglandins. (Am J Pathol 2010, 176:1469–1483; DOI: 10.2353/ajpath.2010.090607)

Recent advances in tumor biology have identified the stroma as an important regulator of carcinogenesis and a potentially valuable therapeutic target. Although interactions between the epithelium and stroma have long been considered to be important in tumor progression, the efficacy of targeting stromal components as a therapeutic strategy has not been established, because the specific regulators of such interactions remain unclear. In addition to endothelial cells, macrophages and fibroblasts¹ are the major stromal components of the tumor microenvironment, and they play key roles in the enhancement of angiogenesis. It has recently been established that bone marrow (BM)–derived hematopoietic cells are the major components of the stroma of tumors, and that they determine the tumor microenvironment^{2–8}; however, the specific factors that enhance the functions of BM-derived precursor cells, and the mechanism of recruitment of these cells during tumor angiogenesis, are not fully understood. Tumor-associated angiogenesis in the tumor stroma is a prerequisite for invasive growth of a tumor larger than 2 to 3 mm in diameter, and then metas-

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tasis occurs. Tumor-associated angiogenesis is caused by a shift in the local balance of proangiogenic and antiangiogenic factors toward the proangiogenic state.^{9–11} This angiogenic switch in the tumor stroma may be important in the control of cancer progression and may be regulated by the recruitment of BM-derived cells.

Chemokines and their receptors play critical roles in leukocyte trafficking during inflammatory processes; however, a growing body of data suggests that a number of chemokines and their receptors also play diverse roles in cancer growth, cancer metastasis, cancer angiogenesis, or modulation of the cancer microenvironment.^{12–15} Preclinical tumor models indicate that some chemokine receptor antagonists can block cancer growth either directly or by altering the cancer stroma.^{16–21} However, there is a need to extend our understanding of the signaling pathways by which chemokines and chemokine receptors facilitate cancer processes. Further, the interactions between the inflammatory mediators and chemokine systems are poorly understood.

Cyclooxygenase (COX)–2 is one of two forms of COX and is expressed at sites of inflammation and malignancies, suggesting that COX-2 inhibition may be useful in the treatment or prevention of inflammatory diseases and various cancers.²² In previous studies, we determined that inhibition of the COX-2/VEGF-dependent pathway suppresses tumor-associated angiogenesis and tumor growth in mice.^{23,24} COX-2-lacking mice were found to be resistant to the development of colorectal neoplasia.²⁵ The same may be true in humans, because epidemiological studies have shown that nonsteroidal anti-inflammatory drugs, the prototypic inhibitors of COX, reduce the risk of several types of cancer.²⁶ The relevant endogenous prostaglandins (PGs) may be PGE₂, which is generated from arachidonic acid and other polyunsaturated fatty acids, in a reaction catalyzed by COX-2.²⁷ Four G protein–coupled receptors, which respond to the PGE₂ are designated subtypes EP1, EP2, EP3, and EP4.²⁸ Stromal tissues in COX-2 inhibitor–treated mice and EP3 receptor knockout mice are very thin and appeared to be pale in color.^{23,24} COX-2 inhibition and EP3 receptor knockout markedly reduce the formation of stromal tissues around the tumors besides the attenuation of angiogenesis, suggesting that COX-2–derived PGE₂ has a crucial role in tumor stroma formation and also in the enhancement of tumor-associated angiogenesis.

In the present study, we tested whether COX-2–derived PGE₂ upregulates the expression of chemokines and their receptors, and further clarified whether the upregulated chemokine systems enhance stromal formation and PG-dependent tumor angiogenesis. We showed that the chemokine systems and PGE₂ may be useful therapeutic targets for cancers that modulate tumor microenvironment.

Materials and Methods

Animals and Cells

Eight-week-old male C57BL/6 wild-type mice, obtained from CLEA Japan Inc. (Tokyo, Japan), were used. In some experiments, EP receptor knockout ($^{-/-}$) mice

(eight- to ten-week-old males) lacking EP1, EP2, EP3, and EP4, were used (for EP4 $^{-/-}$ mice, EP4WT mice were used for comparison) as described previously.^{23,29} These mice were housed in a limited access animal facility. The animal room temperature and relative humidity were set to 25 ± 1°C and 60% ± 5%, respectively. Artificial lighting provided a 24-hour cycle of 12 hours light/12 hours dark (8 AM to 8 PM). All experiments were performed in accordance with the guidelines for animal experiments of the Kitasato University School of Medicine.

Lewis lung carcinoma (LLC) cells were originally isolated from C57BL/6 mice, and we used CRL-1642 (ATCC, Manassas, VA), which are high-tumorigenic and low-metastatic type mouse LLC cells. No metastasis has been shown for experimental 28 days when these LLC cells are subcutaneously transplanted. Fibroblasts (L929) were obtained from Cell Bank, RIKEN Bioresource Center (Ibaraki, Japan). They were cultured in DMEM supplemented with 10% (v/v) FBS and 100 units/500 ml penicillin, all of which were obtained from Gibco BRL, Life Technologies, Inc. (Rockville, MD), at 37°C in 5% humidified CO₂. After harvest, LLC cells were washed three times with PBS solution (PBS; 8 g/L NaCl, 0.2 g/L KCl, 2.9 g/L Na₂HPO₄·12H₂O, and 0.2 g/L KH₂PO₄, pH 7.2) and used in the respective experiments.

Mouse Dorsal Matrigel Implantation Assay

Under light ether anesthesia, mice were subcutaneously injected in the back with 0.25 ml of phenol-red-free Matrigel (Japan BD Biosciences, Tokyo, Japan), diluted 1:1 in saline. The day of implantation was defined as day 0. In the PGE₂ administration experiment, PGE₂ (10 nmol/site/day; Cayman Chemical, Ann Arbor, MI) or the same volume of vehicle (0.2% ethanol) was injected in the center of the Matrigel on the day four to six. In the stromal cell–derived factor (SDF)-1 α administration experiment, recombinant murine SDF-1 α (500 ng/mouse/day; Pepro-Tech GmbH, Hamburg, Germany) or the same volume of saline was subcutaneously injected in the center of the Matrigel on day one to six. In both experiments, mice were euthanized with an excess dose of ether after seven days, and the Matrigel implants were harvested with the surrounding stromal tissue and skin. After trimming, the wet weights of the stroma and Matrigel were measured and prepared for the other assays.

Mouse Micropore Chamber Assay

LLC cells were washed as described above and resuspended in PBS solution at a density of 1 × 10⁸ cells/ml. Thereafter, 1 × 10⁷ LLC cells in 0.1 ml of solution were placed in a round chamber, which consisted of a ring covered with cellulose ester filters (pore size, 0.45 μ m; Millipore, Bedford, MA) on both sides. Then, these chambers were subcutaneously implanted into the dorsum of mice.^{24,30} The day of implantation was defined as day 0. In the experiment in which COX-2 inhibitor was administered, mice were fed diets containing 1000 ppm COX-2 inhibitor (Celecoxib; Pfizer Inc., New York, NY) starting

from day 0 until the end of the experiment. The dose of Celecoxib was approximately 100 mg/kg/day, as estimated from the diets they consumed. The control mice were fed diets without agents. In the CXCR4 neutralizing experiment, CXCR4 neutralizing rat monoclonal IgG antibody, named 2B11 (Japan BD Biosciences, Tokyo, Japan), dissolved in PBS solution at a concentration of 70 µg/ml, was subcutaneously injected into four sites around the chamber, at a dose of 3.5 µg/site (total 14 µg/mouse/day) from day one to six. Similarly, the control mouse group was injected at four sites with normal rat IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA) dissolved in PBS solution, at the same dose. In both experiments, mice were euthanized on day seven, and the stromal tissues that had formed on the micropore chamber were immediately used in assays.

Subcutaneous Tumor Cell Implantation Models

LLC cells were washed as described above and resuspended in PBS at a density of 1×10^7 cells/ml, and 0.1 ml of the resulting suspension was subcutaneously injected into the right flank of mice. The day of inoculation was defined as day 0. In the experiment of COX-2 inhibition, mice of the Celecoxib group were fed diets containing 1000 ppm Celecoxib, starting from day 0 until the end of the experiment. The control mice were fed diets without agents. On days 7, 14, and 21, mice were euthanized with an excess dose of ether, then tumor including stromal tissue was carefully excised. In the experiment of each EP receptor knockout mice, the mice were similarly sacrificed on day 14. The wet weights (mg) and size (mm³) of excised tissue were measured immediately after harvesting.

Bone Marrow Chimeric Model of Green Fluorescent Protein

Bone marrow transplantation (BMT) was performed as reported previously.^{29,31} Briefly, BM cells were obtained by flushing the cavities of freshly dissected femurs, tibias, and pelves of donor male green fluorescent protein (GFP) transgenic mice (a gift from Dr. M. Okabe, Genome Information Research Center, Osaka University, Osaka, Japan) with PBS. The flushed BM cells were dispersed by pipetting and resuspended in PBS at a density of 1×10^7 cells/ml. Wild-type mice were lethally irradiated with 9.0 Gy using an MBR-1505R X-ray irradiator (Hitachi Medico Co., Tokyo, Japan) with a filter (copper, 0.5 mm; aluminum, 2 mm), monitoring the cumulative radiation dose. The BM mononuclear cells of GFP mice (2×10^6 cells in 200 µl of PBS) were transplanted into irradiated wild-type mice via the tail vein. After 12 weeks, peripheral blood was collected and analyzed by fluorescence-activated cell sorting. The mice in which more than 90% of the peripheral leukocytes were GFP-positive were used in the tumor implantation experiment.

On day 14, the GFP-BMT-chimeric mice were euthanized with an excess dose of ether, and tumor and stromal tissue were carefully excised. The wet weight (mg)

and size (mm³) of excised tissue were measured immediately after harvesting. A 50-mg aliquot of excised tissue was homogenized in 1 ml of PBS. The fluorescence of the supernatants (3000g for 30 minutes, at 4°C) was measured in 96-well plates using a 488 nm excitation wavelength.

Effect of PGE₂ and EP-Specific Agonists on Fibroblasts

L929 fibroblasts (3×10^5 cells per well) precultured for six hours in six-well plates were incubated for 24 hours with or without PGE₂ and the respective agonists; EP1 agonist (ONO-DI-004), EP2 agonist (ONO-AE1-259-01), EP3 agonist (ONO-AE-248), and EP4 agonist (ONO-AE1-329; Ono Pharmaceutical Co., Osaka, Japan) as described previously.^{29,32} After harvest, RNA extraction and real-time RT-PCR analysis were performed as described below.

Immunohistochemistry

For histological analysis, excised tissues were immediately fixed with 4% paraformaldehyde in 0.1 mol/L sodium phosphate buffer (pH 7.4) for 24 hours, dehydrated with a graded series of ethanol solutions, and embedded in paraffin. Sections (4 µm thick) were prepared from the paraffin-embedded tissue and mounted on glass slides which had been treated with VECTABOND (Vector Laboratories Inc., Burlingame, CA) according to the manufacturer's instructions. After deparaffinization with xylene and hydration with a degraded series of ethanol solution, the sections were subjected to either H&E staining or immunostaining. For immunostaining, antigen unmasking was performed with Protease-K (DakoCytomation, Glostrup, Denmark) for three minutes, endogenous peroxidase activity was blocked by incubation in 3% hydrogen peroxide/phosphate buffer for five minutes, and non-specific antibody binding was blocked by incubation with 1% diluted normal horse serum for 30 minutes. Sections were then incubated at 4°C overnight with the following antibodies: rat anti-mouse CD31 monoclonal IgG_{2a} antibody (Abcam plc, CB, UK), to identify endothelial cells of the microvessels; mouse anti-mouse SDF-1 monoclonal IgG₁ antibody (P-159X: Santa Cruz Biotechnology, Inc., Santa Cruz, CA), to detect SDF-1α (CXCL12); rat anti-mouse CXCR4 monoclonal IgG_{2b} antibody (2B11: Japan BD Biosciences, Tokyo, Japan), to detect CXCR4; rabbit anti-mouse S100A4 polyclonal IgG antibody (Abcam plc, CB, UK), to identify fibroblasts in the stromal tissue; rat anti-mouse F4/80 monoclonal IgG_{2a} antibody (BM8: Santa Cruz Biotechnology Inc., Santa Cruz, CA) or goat anti-mouse F4/80 polyclonal IgG antibody (A-19: Santa Cruz Biotechnology Inc., Santa Cruz, CA) to identify monocytes; goat anti-mouse CD3ε polyclonal IgG antibody to identify lymphocytes (M-20: Santa Cruz Biotechnology Inc., Santa Cruz, CA), and rabbit anti-mouse α-smooth muscle actin (α-SMA) monoclonal IgG antibody (E184: Abcam plc, CB, UK) or mouse anti-mouse α-SMA monoclonal IgG_{2a} antibody (1A4: Sigma-Aldrich

Inc., St. Louis, MO) to identify myofibroblasts. Immune complexes were detected with a Vectastain Universal *Elite* ABC Kit (Vector Laboratories Inc., Burlingame, CA). Immune complexes of primary antibodies raised in mice were detected with the mouse-on-mouse (M.O.M.) kit (Histofine Mouse Stain Kit, Nichirei Biosciences Inc., Tokyo, Japan), according to the manufacturer's instructions. These immune complexes were revealed using the 3, 3'-diaminobenzidine (DAB) substrate, with nickel ammonium sulfate (Vector Laboratories Inc., Burlingame, CA) as chromogen. Sections were counterstained with hematoxylin.

Immunofluorescence

For double immunofluorescence, samples were fixed with 10% neutral buffered paraformaldehyde at 4°C for 1 hour. After cryoprotection with 30% sucrose/0.1 mol/L phosphate buffer (pH 7.2), cryostat sections about 10 to 20 μm in thickness were cut and nonspecific staining was blocked by the incubation with 1% bovine serum albumin (BSA)/PBS for 1 hour. The sections were incubated with a mixture of primary antibodies in 1% BSA/PBS at room temperature for 1 hour or overnight. Primary antibodies were the same as the above immunohistochemistry. After being washed three times in PBS, the sections were incubated with a mixture of secondary antibodies for 1 hour at room temperature. Negative control staining was conducted by replacing the primary antibodies with control IgG of each immune animal. Secondary antibodies were Alexa Fluor 488, 568, or 647- conjugated donkey anti-mouse, rabbit, rat, or goat IgGs (Molecular Probes, Inc., Eugene, OR). They were then observed using a confocal scanning laser microscope (LSM710: Carl Zeiss MicroImaging GmbH, Oberkochen, Germany). Serial optical sections (collected at 1- μm intervals) in the z axis were collected and overlaid for the final images shown using ZEN-2008 software (Carl Zeiss MicroImaging GmbH, Oberkochen, Germany) installed on LSM710.

After double labeling, four high-power optical fields (400-fold magnification) were randomly selected, and the number of positive cells was counted for every single channel (green, red) as well as the merged channels (yellow) for each animal. At least five animals were analyzed for each marker analysis. All samples were blinded before this morphometric analysis.

Microvessel Density and Microvessel Area

Microvessel density (MVD) and microvessel area (MVA) in the stroma were assessed as a parameter of tumor-associated angiogenesis, according to the established methods described previously.^{24,33} Briefly, areas of highest neovascularization were found by scanning the stroma around the tumor at low power (40-fold and 100-fold magnification). After the most vascularized area was identified, the individual microvessels were then counted on a 400-fold field. CD31 immunoreactive endothelial cells were clearly and easily differentiated from the stromal cells and other connective tissue elements. MVD was

expressed in terms of the number of vessels per unit area (mm^2). MVA was also calculated with the image analysis software National Institutes of Health Image (NIH Research Service Branch) and was expressed as the area of vessels per observed area (mm^2/mm^2). MVD and MVA were calculated in four different fields per section, and four sections were obtained from each animal.

Stromal Thickness

Stromal thickness was assessed on a 100-fold field of an H&E-stained section, calculated in four fields per section, and four sections/individual, and averaged.

Quantitative Real-Time RT-PCR Analysis

Each sample of excised tissue was immediately immersed in RNeasy lysis reagent (QIAGEN Japan, Tokyo, Japan), and the samples were homogenized for 60 seconds at 6000 rpm using a MagNA Lyser (Roche diagnostics Inc., Mannheim, Germany). Harvested cells were washed three times with PBS solution and homogenized using QIA Shredder (QIAGEN Japan, Tokyo, Japan). Total RNA was extracted from the homogenized tissues and cells using the RNeasy Mini Kit (QIAGEN Japan, Tokyo, Japan), and single-stranded cDNA was generated from 1 μg of total RNA via reverse transcription using ReverTra Ace (TOYOBO Co., Ltd., Osaka, Japan), according to the manufacturer's instructions. Quantitative PCR amplification was performed using SYBR[®] Premix Ex Taq (Takara Bio Inc., Shiga, Japan). The gene-specific primers used were described in Table 1. PCR was performed in 20 μL reactions according to the method of the manufacturer. The reaction mixture was then subjected to 35 cycles of amplification in a DNA thermal cycler. Each cycle consisted of a heat-denaturation step at 95°C for 1 minute, an annealing of primers step at 55°C for 1 minute, and an extension step at 72°C for 1 minute. After completion of 35 PCR cycles, the samples were incubated at 72°C for 5 minutes. Data were normalized relative to the expression level of GAPDH for each sample. To confirm that PCR products were derived from target mRNA, the products were cloned using TOPO TA Cloning Kit for Sequencing (Invitrogen, San Diego, CA), and the nucleotide sequences were analyzed. All samples were tested in triplicate.

Measurement of Stromal CXCL12 (SDF-1 α) Protein Level

Subcutaneous stromal tissues surrounding tumors were isolated from the surface of tumors, and tissues were homogenized in ninefold volume (w/v) of PBS with 0.1% (v/v) Triton X, and centrifuged for 30 minutes at 3500g at 4°C. The supernatant was recovered, and CXCL12 (SDF-1 α) protein levels were determined using a mouse SDF-1 α Quantikines ELISA Kit (R&D Systems Inc., Minneapolis, MN). Data are expressed as ng/ml of lysate. All samples in the CXCL12 assay were tested in duplicate.

Table 1. Primers for Real-Time Quantitative PCR

Gene	Forward primer sequence	Reverse primer sequence	Annealing temperature
<i>GAPDH</i>	5'-TTGTGATGGGTGTGAACCAC-3'	5'-CATGAGCCCTTCCACAATGCC-3'	55
<i>CCL2</i>	5'-CGGAACCAAATGAGATCAGAA-3'	5'-TTGTGGAAAAGGTAGTGGATG-3'	55
<i>CCL3</i>	5'-TGACTAAGAGAAACCGGCAGA-3'	5'-TCAGGCATTCAGTTCAGGTC-3'	55
<i>CCL4</i>	5'-TCCTGCTGTTTCTCTTACACC-3'	5'-TGTCTGCCTCTTTTGGTCAGG-3'	55
<i>CCL5</i>	5'-CTGCTGCTTTGCCTACCTCTC-3'	5'-GTGACAAACACGACTGCAAGA-3'	55
<i>CCL6</i>	5'-TCCTTATCCTTGTGGCTGTCC-3'	5'-GCAGCAGTCTGAAGAAGTGTC-3'	55
<i>CCL7</i>	5'-TGCTTTCAGCATCCAAGTGTG-3'	5'-ACCGACTACTGGTGATCCTTC-3'	55
<i>CCL8</i>	5'-CCAGATAAGGCTCCAGTCACC-3'	5'-TTCTCTCGTAGCTTTTCAGCA-3'	55
<i>CCL9</i>	5'-ACAAGTGTCTTGAATCTGG-3'	5'-AGTCTTGAAAGCCCATGTGAA-3'	55
<i>CCL12</i>	5'-CATAGCTACCACCATCAGTCC-3'	5'-TGCTTGTGATTCTCCTGTAGC-3'	55
<i>CXCL1</i>	5'-AAACCGAAGTCATAGCCACAC-3'	5'-GGGGACACCTTTTAGCATCTT-3'	55
<i>CXCL2</i>	5'-ATCCAGAGCTTGAGTGTGACG-3'	5'-GCCTTGCCTTTGTTCAGTATC-3'	55
<i>CXCL3</i>	5'-CCCTACCAAGGGTGTGATTTG-3'	5'-GGCAAACCTCTTGACCATCCT-3'	55
<i>CXCL5</i>	5'-GCCCTACGGTGAAGTCATAG-3'	5'-TGCAATCCGCTTAGCTTTCTT-3'	55
<i>CXCL7</i>	5'-TGCCCACTTCATAACCTCCA-3'	5'-GTCCATGCCATCAGATTTTTC-3'	55
<i>CXCL9</i>	5'-GTTCTTTTCTCTCTGGGCATC-3'	5'-GAGCATCGTGCATTCCTTATC-3'	55
<i>CXCL10</i>	5'-CGTCATTTTCTGCCTCATCCT-3'	5'-CCTATGGCCCTCATTCTCACT-3'	55
<i>CXCL11</i>	5'-TGCTCAAGGCTTCTTATGTT-3'	5'-CTTTCTCGATCTCTGCCATTT-3'	55
<i>CXCL12</i>	5'-GCATCAGTGACGGTAAACCAG-3'	5'-GCACAGTTTGGAGTGTGAGG-3'	55
<i>CCR1</i>	5'-GGCCCTAGCCATCTTAGCTTC-3'	5'-TGAAACCTCTTCCACTGCTTC-3'	55
<i>CCR2</i>	5'-TTACCTCAGTTCATCCACGGC-3'	5'-CAAGGCTCACCATCATCGTAG-3'	55
<i>CCR3</i>	5'-GCAGCATTCCTGAATTTATC-3'	5'-TTTCCAGCTGTCTTCTTACC-3'	55
<i>CCR5</i>	5'-GTTATCTCTCAGTGTCTTCC-3'	5'-ATCGGGTATAGACTGAGCTTG-3'	55
<i>CCR6</i>	5'-TTACCCCTTTGCTGTTTATG-3'	5'-CTGTGCCTCTTGAGTGTCTG-3'	55
<i>CCR7</i>	5'-TACATCGGCGAGAATACCACG-3'	5'-ATACATGAGAGGCAGGAACCA-3'	55
<i>CCR8</i>	5'-CGACCTGCTCTTTGCTCCTCTC-3'	5'-CCAGAGACCACCTTACACATC-3'	55
<i>CXCR2</i>	5'-AACAAATACATCCCGTTTGAGG-3'	5'-AGTGTGAACCCGTAGCAGAAC-3'	55
<i>CXCR3</i>	5'-TGATATGGGTCTCTGTCTGCT-3'	5'-ACCTGTGGGAAGTTGTACTGG-3'	55
<i>CXCR4</i>	5'-CTCTGAAGAAGTGGGGTCTGG-3'	5'-AAGTAGATGGTGGGCAGGAAG-3'	55
<i>CXCR7</i>	5'-TTTGCTGTCCCTTCACTATC-3'	5'-CCAACATACCAGGAAGACCAC-3'	55
<i>CX3CR1</i>	5'-GTGACATGAAGAGGGACCTGA-3'	5'-GGCGTAGATAAAGGGGTTGAG-3'	55
<i>GFP</i>	5'-ACTACAACAGCCACAACGCTC-3'	5'-GGTGGTCTGCTGGTAGTGGTC-3'	55
<i>VEGF-A</i>	5'-ACGACAGAAGGAGAGCAGAAG-3'	5'-ATGTCCACCAGGGTCTCAATC-3'	55
<i>F4/80</i>	5'-TATCTTTTCTCGCTGCTTC-3'	5'-CACCACCTTCAGGTTTCTCAC-3'	55
<i>S100A4</i>	5'-TGGGGAAAAGGACAGATGAAG-3'	5'-ATGCAGGACAGGAAGACACAG-3'	55
<i>α-SMA</i>	5'-GAAGAGCTACGAAGTGCCTGA-3'	5'-TGAAAGATGGCTGGGAAGAGAG-3'	55
<i>CD3ε</i>	5'-ACTGGAGCAAGAATAGGAAGG-3'	5'-ATAGTCTGGGTGGGAACAGG-3'	55

SMA indicates smooth muscle actin.

Statistical Analysis

Statistical computations were performed using a SAS software package (SAS Institute, Cary, NC), StatView version 5.0. Data were expressed as means ± SEM. Comparisons among multiple groups were performed by factorial analysis of variance, followed by Scheffé test. Comparisons between two groups were performed with Student *t* test. A result was considered statistically significant when the *P* value was <5% (*P* < 0.05).

Results

COX-2-Dependent Stromal Formation and Angiogenesis in the Tumor Implantation Model, and Changes in the Expression of Chemokines and Chemokine Receptors in the Tumor Stroma

We first examined the roles of inducible COX-2 in angiogenesis and tumor stromal formation and clarified which chemokines and their receptors were involved in stromal formation. We adopted the tumor implantation model with the use of high-tumorigenic LLC cells. Tumor growth in

mice was observed during three-week experimental periods. Celecoxib administration (100 mg/kg/day) suppressed tumor growth significantly throughout the experimental periods (Figure 1A). As shown in Figure 1B, Celecoxib-treated mice exhibited less evidence of angiogenesis, which was visualized as marked red color, than vehicle-treated mice. Consistent with the reduced redness of the tumors, MVD and MVA were significantly reduced in Celecoxib-treated mice in comparison with those in vehicle-treated mice (Figure 1, C and D). Celecoxib treatment significantly reduced the formation of stromal tissues (Figure 1E). To investigate the involvement of chemokine systems in stromal formation, we next tested the expression of a number of chemokines and their receptors in tumor stromal tissues, using real-time RT-PCR analysis (Table 2). Substantial expression of CCL2, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9, and CCL12 was detected in the stromal tissues throughout the experiment. Celecoxib treatment did not alter the mRNA levels of CCL2, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, and CCL9, whereas it did cause the levels of CCL12 mRNA to decrease in the early periods but to increase on day 14. Further, the mRNA expression of chemokine receptors, CCR1, CCR2, CCR3, CCR5, CCR6,

Figure 1. COX-2-dependent stromal formation and angiogenesis in the tumor implantation model, and expression of chemokines and chemokine receptors in tumor stroma. **A:** Time course of tumor growth in the absence (–; unfilled) and presence (+; filled) of Celecoxib. The day of inoculation was defined as day 0. Celecoxib significantly attenuated tumor growth on days 7, 14, and 21. **B:** Typical appearance of tumors excised on day 14. Scale bars = 5.0 mm. **C and D:** Angiogenesis (MVD and MVA) on day 14. **E:** Stromal formation (stromal thickness) on day 14. **F and G:** The mRNA expression of CXCL12 and CXCR4 in tumor stroma on day 14. **H:** CXCL12 protein expression in tumor stroma on day 14. **C–H:** The results represent means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$ by Student *t* test (compared with vehicle-treated mice on the same day).

CCR7, CCR8, and CX3CR1, did not significantly change with Celecoxib treatment. By contrast, CXCL12 (SDF-1) mRNA levels were reduced by 73% in Celecoxib-treated mice relative to vehicle-treated mice on day 14 (Figure 1F). In line with mRNA expression, CXCL12 protein expression was suppressed by 42% (Figure 1H). Even on day 7, CXCL12 expression tended to be lower in the Celecoxib-treated mice than in the vehicle-control mice (vehicle versus celecoxib, 0.0180 ± 0.0039 and 0.0107 ± 0.0024 , $P = 0.12$), and also at day 21 (0.0095 ± 0.0026 and 0.0055 ± 0.0009 , $P = 0.12$). In addition, the expression of CXCR4, a receptor for CXCL12, was reduced on day 14 (Figure 1G), in Celecoxib-treated mice, suggesting that the CXCL12/CXCR4 axis may be at least in part involved in regulating tumor stromal formation via COX-2 derived PGs. CXCR7, recently orphaned as an alternative receptor that binds CXCL12,³⁴ did not significantly alter in its mRNA expression by Celecoxib-treatment (Table 2). In addition, we also determined the stromal expression of two important CXC chemokine receptors, CXCR2 and CXCR3, because those chemokines also play a role in angiogenesis and angiostasis, respectively.^{15,35} Gene expression of angiogenic CXCR2 and its ligands including CXCL1, CXCL2, CXCL3, CXCL5, and CXCL7 did not alter by COX-2 inhibition throughout the experimental period. Gene expression of CXCR3 did not change, though its ligands including CXCL9, CXCL10, and CXCL11 were significantly increased on day 14 (see Supplemental Table S1 at <http://ajp.amjpathol.org>).

COX-2-Dependent Recruitment of BM Cells to the Tumor Stroma

To evaluate the role of COX-2-derived PGs in the recruitment of BM cells to tumor stroma, we produced BM chimeric mice with GFP-positive BM. LLC cells were implanted subcutaneously after intravenous injections of BM cells isolated from GFP-transgenic mice and reconstitution of the BM. Stromal tissues were isolated on day 14, and GFP fluorescence intensity was determined. Celecoxib treatment significantly reduced the stromal fluorescence intensity (see Supplemental Figure S1A at <http://ajp.amjpathol.org>) and the mRNA levels of GFP significantly (see Supplemental Figure S1B at <http://ajp.amjpathol.org>). Furthermore, we established that the expression of CXCL12 and CXCR4 was reduced on COX-2 inhibition (see Supplemental Figure S1, C and D at <http://ajp.amjpathol.org>). These results suggest that COX-2-derived PGs regulate the recruitment of BM cells to the tumor stroma.

Effect of Topical Application of PGE₂ on Stromal Formation in a Matrigel Implantation Model

To determine the role of PGE₂ in stromal formation, we used the Matrigel implantation model. This model is characterized by the formation of the granulation tissues around the gel implants³⁶ and is designed to mimic tumor-associated angiogenesis. Application of PGE₂ enhanced angiogene-

Table 2. mRNA Expression of Chemokines and Chemokine Receptors in the Stroma of Murine Tumor Model

mRNA	Celecoxib	Day 7	P*	Day 14	P*	Day 21	P*
CCL2	–	0.2850 ± 0.1070	NS	0.1810 ± 0.0434	NS	0.1680 ± 0.0429	NS
	+	0.1370 ± 0.0375		0.2180 ± 0.0553		0.2940 ± 0.0258	
CCL3	–	0.0126 ± 0.0076	NS	0.0220 ± 0.0120	NS	0.0380 ± 0.0181	NS
	+	0.0131 ± 0.0052		0.0138 ± 0.0009		0.0211 ± 0.0090	
CCL4	–	0.0035 ± 0.0003	NS	0.0044 ± 0.0013	NS	0.0063 ± 0.0012	NS
	+	0.0041 ± 0.0012		0.0078 ± 0.0028		0.0080 ± 0.0021	
CCL5	–	0.0178 ± 0.0055	NS	0.0075 ± 0.0016	NS	0.0103 ± 0.0038	NS
	+	0.0064 ± 0.0016		0.0070 ± 0.0019		0.0269 ± 0.0183	
CCL6	–	1.090 ± 0.3670	NS	0.7200 ± 0.5670	NS	0.2430 ± 0.0729	NS
	+	0.3590 ± 0.0714		0.3300 ± 0.2270		0.2470 ± 0.0959	
CCL7	–	0.0196 ± 0.0060	NS	0.0268 ± 0.0078	NS	0.0319 ± 0.0125	NS
	+	0.0184 ± 0.0061		0.0283 ± 0.0106		0.0257 ± 0.0093	
CCL8	–	0.9800 ± 0.4190	NS	0.3420 ± 0.2400	NS	0.1800 ± 0.0780	NS
	+	0.1970 ± 0.0601		0.2540 ± 0.1610		0.1790 ± 0.1220	
CCL9	–	0.2540 ± 0.0725	NS	0.1820 ± 0.1270	NS	0.0867 ± 0.0306	NS
	+	0.0928 ± 0.0099		0.0902 ± 0.0453		0.0976 ± 0.0232	
CCL12	–	0.0339 ± 0.0046	0.03	0.0281 ± 0.0035	NS (0.07)	0.0251 ± 0.0030	NS
	+	0.0201 ± 0.0016		0.0432 ± 0.0054		0.0228 ± 0.0017	
CCR1	–	0.0216 ± 0.0036	NS	0.0200 ± 0.0022	NS	0.0158 ± 0.0010	NS
	+	0.0128 ± 0.0021		0.0158 ± 0.0013		0.0133 ± 0.0007	
CCR2	–	0.0125 ± 0.0005	NS	0.0114 ± 0.0023	NS	0.0071 ± 0.0008	NS
	+	0.0098 ± 0.0014		0.0141 ± 0.0034		0.0047 ± 0.0007	
CCR3	–	0.0008 ± 0.0003	NS	0.0004 ± 0.0001	NS	0.0006 ± 0.0002	NS
	+	0.0010 ± 0.0004		0.0010 ± 0.0006		0.0005 ± 0.0005	
CCR5	–	0.0358 ± 0.0169	NS	0.0215 ± 0.0032	NS	0.0082 ± 0.0008	NS
	+	0.0108 ± 0.0031		0.0290 ± 0.0028		0.0085 ± 0.0018	
CCR6	–	0.0002 ± 0.0001	NS	0.0003 ± 0.0001	NS	0.0001 ± 0.0000	NS
	+	0.0002 ± 0.0000		0.0003 ± 0.0001		0.0001 ± 0.0000	
CCR7	–	0.0015 ± 0.0002	NS	0.0014 ± 0.0004	NS	0.0032 ± 0.0025	NS
	+	0.0011 ± 0.0006		0.0036 ± 0.0007		0.0007 ± 0.0002	
CCR8	–	0.0001 ± 0.0001	NS	0.0002 ± 0.0001	NS	0.0001 ± 0.0000	NS
	+	0.0001 ± 0.0000		0.0002 ± 0.0001		0.0001 ± 0.0000	
CXCR7	–	0.0038 ± 0.0004	NS (0.07)	0.0104 ± 0.0061	NS	0.0027 ± 0.0004	NS
	+	0.0081 ± 0.0019		0.0080 ± 0.0035		0.0021 ± 0.0009	
CX3CR1	–	0.0005 ± 0.0002	NS	0.0010 ± 0.0004	NS	0.0009 ± 0.0003	NS
	+	0.0002 ± 0.0001		0.0030 ± 0.0017		0.0004 ± 0.0001	

NS indicates not significant. Data are means ± SEM.

*Compared with vehicle-treated mice on the same day by Student *t* test.

sis, as gauged by measurements of MVD (see Supplemental Figure S2A at <http://ajp.amjpathol.org>) and MVA (see Supplemental Figure S2B at <http://ajp.amjpathol.org>), and stromal thickness (see Supplemental Figure S2C at <http://ajp.amjpathol.org>). PGE₂ also increased the expression of CXCL12 and CXCR4 (see Supplemental Figure S2, D and E at <http://ajp.amjpathol.org>). These results imply that COX-2–derived PGE₂ may enhance stromal formation and angiogenesis.

COX-2–Dependent Enhancement of Stromal Formation and Angiogenesis around Micropore Chambers, and the Involvement of CXCL12/CXCR4 Signaling

To evaluate stromal reactions without infiltration of tumor cells, we used micropore chambers filled with LLC cells, which allowed the secretion of soluble factors from LLC cells, and the separation of stromal tissues from LLC cells. We first tested the effects of COX-2 inhibition on tumor-associated stromal formation. The administration of Celecoxib strongly reduced granulation tissue formation, mimicking the tumor stromal reactions, and was accompanied by suppressed angiogenesis as determined by MVD and

MVA around the LLC-containing chambers (Figures 2, A, B, and C). As shown in Figure 2, D and E, Celecoxib suppressed the formation of granulation tissues on day 14. COX-2 inhibition reduced the mRNA expression of CXCL12 (Figure 2F) and CXCR4 (Figure 2G) in the granulation tissues. An ELISA assay for CXCL12 revealed that immunoreactive CXCL12 levels in the granulation tissues were reduced under COX-2 inhibition (Figure 2H). The levels of VEGF-A were also reduced when COX-2 was inhibited (Figure 2I). Immunohistochemical examinations revealed that cells positive for CXCL12 or CXCR4 may have been mononuclear cells resembling macrophages or fibroblasts, and that the populations of CXCL12- and CXCR4-positive cells had been markedly reduced in granulation tissues by COX-2 inhibition (see Supplemental Figure S3, A–D at <http://ajp.amjpathol.org>). These results suggest that COX-2 facilitates not only tumor-associated angiogenesis, but also stromal formation, and that the CXCL12/CXCR4 axis may be involved in these effects.

Next, we performed immunohistochemistry and immunofluorescence of major components (fibroblasts, monocytes, and lymphocytes) in stromal cells to identify the cell type that has pivotal role in tumor stromal formation. Especially, fibroblasts are thought to be the main effector cells in the

Figure 2. COX-2-dependent enhancement of stromal formation and angiogenesis around micropore chambers, and the involvement of CXCL12/CXCR4 signaling. **A:** Typical appearance of granulation tissues formed around micropore chambers. Diameter of chamber ring, 13 mm. **B and C:** Angiogenesis formed around micropore chambers, as measured by MVD and MVA. **D and E:** Stromal wet weight and stromal thickness. **F, G and H:** mRNA expression of CXCL12, CXCR4, and VEGF-A, respectively. **H:** CXCL12 protein level in granulation tissues formed around micropore chambers. **J:** Immunofluorescence analysis of CXCL12 (left) and S100A4 (middle) expression in tumor stroma from vehicle-treated mice (top row) and Celecoxib-treated mice (bottom row). Almost all (99.7%) of CXCL12⁺ cells are positive for S100A4; arrows; merged signal, yellow (right). Arrowheads indicate single labeled cells with S100A4. Scale bars = 20 μ m. Quantification of each labeled cells is shown. Treatment with Celecoxib decreased the number of CXCL12⁺S100A4⁺ cells. **K:** mRNA expression of S100A4, F4/80, and CD3e in granulation tissues. The results represent means $\pm$ SEM from 10 mice per group. * P < 0.05; ** P < 0.01 by Student t test (compared with vehicle-treated mice).

stromal compartment for cancer cell progression and metastasis.^{37,38} α -SMA has been used as a marker of cancer-associated myofibroblasts, which are more competent in enhancing tumor progression³⁹; however, the uncertainty in defining the myofibroblasts has been suggested.⁴⁰ On the other hand, S100A4 expression is increased in activated fibroblasts and myofibroblasts and has been used as a highly specific marker of those cells,^{41–43} and moreover, S100A4 per se contributes to tumor progression.⁴⁴ In fact, S100A4 expression was observed in more fibroblasts of normal tissues than α -SMA (see Supplemental Figure S4A at <http://ajp.amjpathol.org>). Analyzed by double labeling of S100A4 and α -SMA, most fibroblasts were S100A4⁺ α -

SMA⁺ (79.6 $\pm$ 3.1%) in the micropore chamber model, whereas only 0.3 $\pm$ 0.2% fibroblasts were single positive for α -SMA, as depicted in Supplemental Figure S5 (see Supplemental Figure S5 at <http://ajp.amjpathol.org>). These fibroblasts lineage rate was not altered, though total numbers of fibroblasts were decreased by COX-2 inhibition. These results suggest that S100A4-positive cells represented tumor stromal fibroblasts in this study. Celecoxib treatment significantly decreased the expression of the S100A4 gene in the granulation tissues, whereas the expression of F4/80 and CD3e (markers for monocytes and lymphocytes, respectively) was not affected by COX-2 inhibition (Figure 2K). In fact, the numbers of fibroblasts in stromal tissues that

Figure 3. A–R: Double labeling analysis of CXCL12 or CXCR4 with S100A4, F4/80, or CD3e in tumor stroma 14 days after tumor implantation. Among the CXCL12-positive cells in the tumor stroma, 94.4% are S100A4-positive (**A–C**), 6.4% are F4/80-positive (**G–I**), and 0% are CD3e-positive (**M–O**). Among CXCR4-positive cells, 87.8% are S100A4-positive (**D–F**), 5.8% are F4/80-positive (**J–L**), and 0% are CD3e-positive (**P–R**). Most CXCL12⁺ or CXCR4⁺ stromal cells were S100A4-positive fibroblasts. **S–U:** Double labeling assay of GFP and S100A4 in tumor stroma of tumor implantation model in GFP-BM chimeric mice. 92.3% of GFP⁺ cells were positive for S100A4. And GFP⁺S100A4⁺ cells occupied 89.6% of S100A4-positive stromal fibroblasts. Scale bars = 20 μ m.

stained with the S100A4 antibody were declined on COX-2 inhibition, as observed by immunohistochemical analysis (see Supplemental Figure S3, E and F at <http://ajp.amjpathol.org>). Because immunohistological studies suggested that cells positive for CXCL12 or CXCR4 may have been mononuclear cells resembling fibroblast, we performed double immunofluorescence of CXCL12 and S100A4. Interestingly, double labeling assay of CXCL12 and S100A4 revealed that almost all (99.7%) CXCL12-positive cells were S100A4-positive fibroblasts, and the numbers of CXCL12⁺S100A4⁺ stromal cells were significantly decreased by COX-2 inhibition (Figure 2J). These results suggest that CXCL12/CXCR4 signaling recruits fibroblasts in a COX-2-dependent manner and thereby has a role in tumor-associated stromal formation.

Most Fibroblasts in Tumor Stroma Were Recruited from BM and Predominantly Produced CXCL12

To identify the cells that express CXCL12 and CXCR4, we also used tumor implantation model ($n = 5$). Tumor and stromal tissue were excised 14 days after the inoculation of LLC cells. Double labeling of CXCL12 and each cell marker (S100A4, F4/80, and CD3e) showed that fibroblasts pre-

dominantly expressed CXCL12 and CXCR4 in the stroma ($94.4 \pm 1.5\%$ of CXCL12-positive cells and $87.8 \pm 2.5\%$ of CXCR4-positive cells) in accordance with the above micro-pore chamber model (Figure 3, A–R).

Similarly, we next evaluated tumor implantation model in GFP-BM chimeric mice to discriminate stromal fibroblasts whether they are recruited from BM or not ($n = 4$). Interestingly, GFP⁺S100A4⁺ cells reached $89.6 \pm 1.0\%$ of S100A4-positive stromal fibroblasts, suggesting that most tumor stromal fibroblasts were recruited from BM (Figure 3, S–U). These results suggest that most fibroblasts expressing CXCR4 are recruited from BM to tumor stroma and produce CXCL12, leading to further recruitment of stromal fibroblasts.

SDF-1 α (CXCL12) Enhances Stromal Formation in a Matrigel Implantation Model

To confirm that CXCL12 has a role in enhancement of stromal formation and angiogenesis, we exposed Matrigel implants to topical injections of CXCL12 (SDF-1 α). CXCL12 injections enhanced granulation tissue formation mimicking the stromal formation and angiogenesis (Fig-

Figure 4. CXCL12 (SDF-1 α) enhances granulation tissue formation that mimics the stromal reaction and angiogenesis in a Matrigel implantation model. **A:** Typical appearance of stromal formation and angiogenesis formed around a Matrigel. Scale bars = 10 mm. **B** and **C:** Angiogenesis (MVD and MVA) was significantly promoted by CXCL12 application. **D:** Stromal thickness was markedly increased by CXCL12 application. **E:** mRNA expression of VEGF-A in stroma formed around a Matrigel. The results represent means $\pm$ SEM from 11 mice per group. * $P < 0.05$; ** $P < 0.01$ by Student *t* test (compared with vehicle-treated mice).

ure 4, A, B, C, and D) and upregulated the expression of VEGF-A (Figure 4E).

CXCR4 Neutralizing Antibody Reduced Angiogenesis and Granulation Tissue Formation around Micropore Chambers

We further tested the effect of topical injections of the neutralizing antibody against CXCR4 (2B11) around a micropore chamber containing LLC cells. The CXCR4 antibody markedly suppressed stromal formation and angiogenesis (Figure 5, A, B, C, D, and E). The expression of VEGF-A mRNA in the stroma was also suppressed by 2B11 (Figure 5F). The stromal expression of S100A4 gene was significantly down-regulated by 2B11 compared with control IgG, whereas expressions of the F4/80 and CD3 ϵ genes were not influenced (Figure 5, G, H, and I). These results suggest that CXCR4 signaling has a crucial role in tumor-associated stromal formation and angiogenesis in this model.

Figure 5. CXCR4-neutralizing antibody (2B11) reduces angiogenesis and granulation tissue formation around micropore chambers. **A:** Typical appearance of stromal formation and angiogenesis formed around micropore chambers. Diameter of chamber ring, 13 mm. **B** and **C:** Angiogenesis (MVD and MVA) in the granulation tissues around micropore chambers. **D** and **E:** Stromal formation (stromal wet weight and stromal thickness). **F:** mRNA expression of VEGF-A in the granulation tissues. **G, H** and **I:** mRNA expression of S100A4 (**G**), F4/80 (**H**), and CD3 ϵ (**I**) in granulation tissues. $n = 11$ in IgG-treated group and $n = 15$ in 2B11-treated group. The results represent means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Student *t* test (compared with control IgG-treated mice).

Identification of the EP Receptor Signaling that Facilitates Stromal Formation in the Tumor Implantation Model

We previously showed that COX-2/PGE₂ promotes tumor angiogenesis and tumor growth.^{23,24} In the present study,

Figure 6. Identification of EP receptor signaling pathways that facilitate of stromal formation in the tumor implantation model. **A** and **B**: Stromal wet tissue weight and stromal thickness in each EP receptor knockout mice. **C** and **D**: mRNA expression of CXCL12 and CXCR4 in stromal tissue in each EP receptor knockout mice. **E**: CXCL12 protein level in stromal tissue of wild-type, EP3^{-/-}, and EP4^{-/-} mice. **F**: mRNA expression of S100A4 in stromal tissue in EP receptor knockout mice. *n* = 10, 4, 5, 8, 8, and 7 in wild-type (WT), EP1^{-/-}, EP2^{-/-}, EP3^{-/-}, EP4WT, and EP4^{-/-}, respectively. The results represent means ± SEM. **P* < 0.05; ***P* < 0.01 by Student *t* test (compared with wild-type mice in EP1, 2, and 3^{-/-} mice, EP4WT mice were used for comparison as to EP4^{-/-} mice).

we identified the EP receptors (EP1, EP2, EP3, and EP4) responsible for stromal formation in tumor implantation model. Fourteen days after tumor inoculation, tumor stromal formation was significantly suppressed in EP3^{-/-} mice and EP4^{-/-} mice, whereas disruption of either EP1 or EP2 did not affect tumor stromal formation (Figure 6, A and B). The expressions of CXCL12 and CXCR4 genes (Figure 6, C and D) and of CXCL12 protein (Figure 6E) in stromal tissues were suppressed in EP3^{-/-} mice and in EP4^{-/-} mice as confirmed immunohistochemically in Supplemental Figure S6, A–F at <http://ajp.amjpathol.org>. The expression of S100A4 gene was significantly suppressed in EP3^{-/-} and EP4^{-/-} mice, but not in EP1^{-/-} and EP2^{-/-} mice (Figure 6F). The populations of S100A4-positive cells were sparse in tumor stroma in EP3^{-/-} mice and EP4^{-/-} mice, compared with wild-type mice (see Supplemental Figure S6, G–I at <http://ajp.amjpathol.org>). These results suggest that endogenous COX-2-derived PGE₂ may enhance tumor stromal formation by increasing the recruitment of fibroblasts via the CXCL12/CXCR4 axis, which is modulated by EP3/EP4 signaling.

EP3 or EP4 Specific Agonist Stimulates CXCL12 Expression by Fibroblasts

We evaluated the effect of PGE₂ and each EP specific agonists on CXCL12 expression by fibroblasts. PGE₂ at 1

to 100 nmol/L markedly stimulated CXCL12 gene expression by fibroblasts (Figure 7). Similarly, either EP3 or EP4 specific stimulation significantly increased CXCL12 expression as the extent of PGE₂ effects, whereas neither EP1 nor EP2 stimulation did. These results suggest that CXCL12 induction elicited by endogenous PGE₂ is mediated by both EP3 and EP4 receptors on fibroblasts.

Figure 7. Effects of EP1, EP2, EP3, and EP4 specific agonists on CXCL12 gene expression by L929 fibroblasts. L929 fibroblasts (3×10^5 cells per well) cultured in six-well plates were incubated for 24 hours with the respective agonists, then CXCL12 gene expression was measured. Data were expressed as the mean ± SEM of three independent experiments. **P* < 0.05; ***P* < 0.01; ****P* < 0.001 by Student *t* test (compared with vehicle-treated cells).

Discussion

In the present study, we have shown that COX-2–derived PGE₂ upregulates the expression of chemokines and their receptors, CXCL12-CXCR4. Furthermore, we established that the upregulation of this chemokine system enhanced stromal formation, in which fibroblast recruitment was predominantly regulated by COX-2–derived PGE₂, and facilitated tumor-associated angiogenesis. These effects were mediated by EP3/EP4 signaling in the host tissues. Thus, we propose that the chemokine systems, CXCL12-CXCR4, together with COX-2 and the EP receptors, may be used as effective therapeutic targets to treat cancer.

Growth of carcinomas, the most common types of human cancer, is known to be regulated by the tumor microenvironment, which consists of extracellular matrix and a variety of cells, including inflammatory cells, endothelial cells, and fibroblasts, which can generate or release various cytokines and growth factors.⁴⁵ In previous studies, we showed that COX-2/PGE₂ signaling is crucial for tumor angiogenesis, and that this signaling was transduced through the EP3 receptor on the host stromal cells rather than on the tumor cells.²³ Moreover, the stromal cells expressing EP3 receptors, which were recruited from BM, have a significant role in enhancing angiogenesis and tumor growth.³¹ One of the most striking phenotypes of tumors treated with COX-2 inhibitor, and also of tumors in EP3 receptor knockout mice, is a reduced formation of tumor stromal tissues around tumors. These results suggest that COX-2 and host signaling of EP receptors may regulate the stromal tissue formation that constitutes the tumor microenvironment, and that the microenvironment could affect tumor-associated angiogenesis and tumor growth in a PG-dependent manner. Indeed, the tumor implantation model in GFP-BM chimeric mice revealed that recruitment of BM cells around the tumors was dependent on COX-2 activity (see Supplemental Figure S1 at <http://ajp.amjpathol.org>). These results allow us to test the involvement of chemokines and their receptors in tumor stromal formation, which determines the tumor microenvironment.

Chemokines were initially characterized as being chemoattractants for leukocytes that infiltrate into inflamed tissues^{46,47}; however, recent works have demonstrated novel roles for chemokines and their receptors in regulating various aspects of the transformed phenotype, such as tumor growth, angiogenesis, invasion, and metastasis. Here, we tested the involvement of chemokines and chemokine receptors in tumor stromal formation in relation to tumor–stroma interactions which regulate tumor growth and tumor-associated angiogenesis. We characterized the expression of several chemokines and their receptors in tumor stromal tissues after inoculation of LLC cells (Table 2). Substantial expression of CCL2, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9, and CCL12 was detected in the stromal tissues, but COX-2 inhibition did not alter the mRNA levels of these chemokines. The CCR1, CCR2, CCR3, CCR5, CCR6, CCR7, CCR8, and CX3CR1 receptors were detected in tumor stromal tissues, and a COX-2 inhibitor did not signifi-

cantly alter their expression pattern. In the present study, we found that the expression of CXCL12 and its receptor CXCR4 was reduced on COX-2 inhibition, suggesting that the CXCL12/CXCR4 axis may be involved in the regulation of tumor stromal formation by COX-2–derived PGs. Additionally, the gene expression of CXCR7, an alternative receptor for CXCL12, tended to increase only at early time point (Table 2). Recent data have suggested that CXCR7 expression on cancer cells may promote tumor progression and metastasis.^{34,48,49} On the other hand, CXCR7 may impair CXCR4 in its ability to activate G proteins after CXCL12 binding and modulate CXCL12-mediated chemotaxis by heterodimerization with CXCR4.⁵⁰ Thus, a role of CXCR7 in the recruitment of stromal cells also needs to be assessed in future studies. Besides, CXC chemokines containing an ELR motif (glutamic acid-leucine-arginine) are thought to promote angiogenesis, whereas CXC chemokines lacking this sequence are considered to be angiostatic (with the exception of CXCL12).^{15,35} In the present study, the gene expressions of angiostatic CXCR3 chemokine family were increased by COX-2 inhibition, whereas those of angiogenic CXCR2 chemokine family were not affected. These results may imply suppressive COX-2 activity against angiostatic CXCR3 chemokine family in tumor stroma, although further studies are needed.

In the present study, we used a micropore chamber filled with LLC cells to further investigate how BM cells are recruited to form the stroma. With this model, one can separate the stromal tissues from the tumor cells and avoid contamination of tumor cells in the tested sample of stromal tissues. We clarified that endogenous COX-2–derived PGs regulated tumor stromal formation via the CXCL12/CXCR4 axis. Recruitment of fibroblasts was regulated by COX-2–derived PG in this chamber model. Fibroblasts are the major components of stromal tissues in various cancers.^{51–53} Although fibroblasts are known to produce growth factors that influence cancer cell progression, they had been thought to have a passive role in cancer progression, responding to signals from cancer cells. However, it has recently been reported that tumor stromal fibroblasts, named cancer-associated fibroblasts, are crucial for cancer progression,^{51,54,55} and, moreover, may be potential inducers of certain carcinomas.⁵⁶ Epidemiological analysis revealed that elevated levels of stromal fibroblasts are correlated with a poor prognosis for human carcinoma patients.⁵⁷ Therefore, modifications in the stromal fibroblasts may regulate the overall development of cancer.⁵⁴ Cancer-associated fibroblasts have been reported to originate partly from BM-derived progenitor cells.^{58–60} α -SMA has been used as a marker of cancer-associated myofibroblasts, which are major component of cancer-associated fibroblasts,³⁹ although the uncertainty in defining the myofibroblasts exists.⁴⁰ We used S100A4 as a marker of tumor stromal fibroblasts in this study. S100A4 expression is increased in activated fibroblasts and myofibroblasts and has been used as a highly specific marker of those cells,^{41–43} and moreover, S100A4 per se causes tumor progression.⁴⁴ Intriguingly, most fibroblasts positive for α -SMA were also S100A4-positive, and the rate of fibroblast lineages

(α -SMA⁺/S100A4⁺) was not altered by COX-2 inhibition notwithstanding total fibroblasts were decreased. These results suggest that S100A4 may be a good marker for tumor-associated activated fibroblast in tumor stroma. In the present study, COX-2/PGE₂ was found to regulate the recruitment of stromal cells from BM, which was mainly composed of fibroblasts that expressed CXCR4 and producing CXCL12, resulting in tumor growth.

The principal COX-2 product in tumors is PGE₂, which acts through four G protein-coupled receptors designated EP1, EP2, EP3, and EP4.^{61,62} In the present study, EP3 and EP4 receptor signaling was found to be crucial for tumor stromal formation and tumor growth. We previously considered the AP-1-dependent upregulation of VEGF to be important in tumor-associated angiogenesis mediated by the EP3 receptor, because some splicing variants of EP3 that link to the activation of adenylate cyclases may result in tumor-associated angiogenesis²³; however, the EP3 receptor also activates the Raf and MAPK pathway.⁶³ EP4 receptor signaling is known to increase cAMP levels in some types of cells.²⁸ As shown in this study, EP3 and EP4 receptors have similar roles in the enhancement of stromal formation. Thus, the activation of adenylate cyclase through binding to G proteins stimulates cAMP production and, in turn, activation of cAMP-dependent protein kinase A may be involved in tumor stromal formation.⁶⁴ EP4 receptor also activates Akt and ERK-1/2.⁶⁵ Further, EP4 agonist-induced cancer cell migration is known to be regulated by ERK,^{66,67} suggesting that tumor stromal formation may also be regulated by ERK activation. CXCR4 expression was reported to be upregulated at least in part by a cAMP-responsive element in human T lymphocytes.⁶⁸ ERK activation was reported to induce CXCL12 and CXCR4 in neurons.⁶⁹ Thus, ERK may enhance EP3/EP4-dependent stromal formation.

Stimulation of VEGF and bFGF was reported to increase the expression of CXCR4 on endothelial cells. Further, PGE₂ has been shown to mediate the effects of bFGF and VEGF by upregulating CXCR4 expression in human microvascular endothelial cells.⁷⁰ These findings indicate that PGE₂ is a mediator of VEGF- and bFGF-induced CXCR4-dependent neovessel assembly *in vivo* and show that the angiogenic effects of PGE₂ require CXCR4 expression.⁷⁰ In the present study, the recruitment of fibroblasts expressing CXCL12 and CXCR4 to the tumor stroma was found to have a significant role in enhancing angiogenesis and tumor growth. CXCL12 may act on the fibroblasts in an autocrine or paracrine fashion in tumor stroma. PGE₂-EP3/EP4 signaling in tumor stroma may promote tumor stromal formation and tumor growth mainly through the host CXCL12/CXCR4 axis by recruiting stromal fibroblasts.

In conclusion, PGE₂-EP3/EP4 signaling appears to be crucial for tumor-associated stromal formation and tumor growth. EP3 and EP4 signaling on the stromal fibroblasts was correlated with the induction of a potent chemotactic and proangiogenic cytokine, CXCL12, in stromal fibroblasts. The subsequent upregulation of CXCL12/CXCR4 signaling facilitated tumor stromal formation by accelerating the recruitment of fibroblasts,

which resulted in tumor growth. Highly selective EP3, EP4, and CXCR4 antagonists may therefore serve as novel therapeutic tools to treat cancer. These results may elucidate how nonsteroidal anti-inflammatory drugs prevent tumor development.

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